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New ways from the liberal arts?

The University of Chicago, once a prolific source of academic innovation, is charting a new course. Will it have the courage to follow its own prescription? And is that radical enough?

Like many other private universities strong in the liberal arts, the University of Chicago is worried about its future. One problem is simply demographic; the decline of the birth-rate that set in in the 1960s is about to bite. But Chicago is being hurt by other trends — the flight from language preparation in secondary schools in the United States in the past fifteen years for example. One curious consequence is that Chicago, traditionally strong in foreign-language study, has found that its students are more interested in exotic languages than in more common languages such as French and German. The result is that even at the graduate student level, demands on academic resources are accentuated and that telephone calls or access to photocopying machines seem even more like luxuries than they are already in hard-pressed humanities and social science departments. What is to be done?

The university's own solution, hammered out in the past two years by an internal committee appointed by president Hannah Holborn Gray, is that Chicago must radically restructure its higher degrees in the liberal arts if it is to hold its own in the years ahead. The committee rightly deplores the trend to specialization in the liberal arts, the humanities and the social sciences, which has had the effect of locking up graduate students in one-track lines of study for up to eight years. When academic jobs, at Chicago or elsewhere, are quickly drying up, it is no wonder that graduate students are increasingly shy of such courses. The committee's remedy, to turn the courses leading to the master's degree into broad yet rigorous programmes of study that would prepare people for the effective pursuit of intellectual interests in the real world outside the university, is sensible. High time, it may be thought, that students of Spanish should know something of the politics of Central or South America, or that social science students should profit from the strengths of Chicago's professional schools, business and law for example. The committee is also right to insist that such courses can be academically and educationally worthwhile, not mere trash baskets for the output of Chicago's spare teaching capacity, and to offer as an example the masters' programme at Harvard's Kennedy School of Government. What it cannot, of course, promise is the Chicago faculty's willingness to collaborate with this ambitious plan. President Gray is behind the plan, but the departmental baronies have yet to declare themselves.

Making a virtue of necessity is now common among academics. The committee's dictum that "leanness (which in the United States is often perceived as meanness) is also opportunity", fits neatly into this tradition. But there are also good educational grounds for asking that students following doctoral courses in the liberal arts and the humanities should not be allowed to rattle around like lost souls, but should work in research institutes that would force them to rub shoulders with disciplines other than their own and also give them a chance to learn skills other than those of simple scholarship. This is potentially a valuable suggestion, a kind of regrafting of the old German notion of the Humboldt university onto a system of graduate education originally fashioned, in the United States, on that model. Whether it is necessary, or even desirable, to restrict these courses, as the committee suggests, to students who will become academics is another matter. Why not see what the market provides?

Internally, these recommendations will seem to many like a recipe for revolution, but others will be asking whether they go far

enough. The rumpus caused last year by Mr Stephen White's tract called *The New Liberal Arts* has not yet died down. White's argument that a liberal arts education is no longer complete if mathematics and computer language are neglected sent waves of panic round liberal arts establishments, some of which have nevertheless been converted by the hunks of money that the Sloan Foundation has handed out to thirty liberal arts colleges (none of them a research university in the Chicago class). Nobody would pretend that the "new liberal arts" recipe is always best, but Chicago would surely be well placed to follow some such route towards a marriage of the two cultures. The biggest disappointment of Chicago's own proposals for reform is that, while coming out strongly against narrow specialization, they fail even to flirt with this adventurous remedy.

No to Lords by a lady

The British government has lost an opportunity to improve its administration of research.

The perennial question of how the British government should improve its administration of research and development is endlessly boring but nevertheless important. These are some reasons why. Last week, the British government found itself having to tell an international conference in Stockholm that it would spend more on acid rain research, thus restoring funds to a programme recently truncated. The British agriculture ministry, which has for several years used cyanide gas to kill off badgers thought to harbour bovine tuberculosis, has shamefacedly retreated from its policy in the face of evidence that the technique is inhumane. Within the past three years, the mechanism for supporting university research (dually, by grant-making research councils and by general subsidy of universities) has collapsed and nobody knows how it should be resuscitated. The committees that judiciously decide whether this or that research organization should have fractionally more or less to spend next year will cheerfully shrug off responsibility for these disasters, major and minor. For has not the dual-support system collapsed under economic adversity? And surely the difficulties about badgers and acid rain are too tiny to be allowed to bother busy and important people?

This insouciance is mistaken, as is the complacency running through the government's (Cmnd 8591, HMSO, £1.65) reply last week to the modest proposals for change produced last year by the House of Lords Select Committee on Science and Technology (see *Nature* 294, 503; 1981). The trouble with what passes for science policy (and one of the reasons why it is so boring) is the customary separation of the process from its content, the medium from the message.

Faced with a set of recommendations which, far from being radical, were guilefully designed to be compatible with present arrangements, the government has countered with even more modest proposals of its own. Yes, it says, the two principal advisory committees function less effectively than they should, but a little committee-engineering can easily fix that. If the Advisory Board for the Research Councils has let its concern for the uncontentious partitioning of funds among its member research councils take precedence over what should have been its



interest in the health of research, then let it have an extra civil servant to strengthen its secretariat. And if the Advisory Council for Applied Research and Development has failed to take a broad look at its field of interest, why not simply broaden its terms of reference and strengthen its secretariat? In thus rejecting the House of Lords case for a central body to oversee "the whole of scientific endeavour", the government laconically accepts that the present divisions of responsibility will continue.

The few positive parts of the government's response are that Dr Robin Nicholson, the member of the Cabinet Office with responsibility for science and technology should now (not before time) have half a dozen people to help him, that he will become the chairman of a new committee composed of chief scientists from government departments and that there will be an internal but formal annual review of government research based on documents supplied by the separate ministries. This proposal falls short of the House of Lords demand that these reviews should be made public and, even more seriously, does not of itself meet the need that there should be an opportunity for some person or group acting on behalf of the government as a whole to ask searching questions about research of the constitutionally autonomous ministries of which the British government consists. On the face of what the government proposes, there will even now be no regular opportunity for demanding that the Department of Education, in its scramble to save money on higher education, should not further undercut the dual-support system on which the Prime Minister's pledge to "protect" basic research depends, or that the agriculture ministry should make sure that cyanide kills badgers humanely before embarking on its widespread use.

Defence research will similarly remain a rogue elephant, dominating the British government's spending on research and development but designed (with perfect constitutional propriety) primarily to meet the needs of the armed services, and with hardly a thought for industry. The government is hoping that the ubiquitous Dr Nicholson and his helpers (still to be found) will be able to put a foot in this door by his intended membership of two important defence research committees. The puzzle is that the government has not seized this opportunity to devise machinery more fully to integrate defence research with the rest of what it does. People in Whitehall set great store by committee membership, and certainly to be excluded from a key committee is to have no influence of any kind over its affairs. But Whitehall also knows that a lone voice on a committee can be silenced or, worse, made to seem to acquiesce. Especially when (as now) the people principally concerned are more receptive than ever to the notion that the benefits of defence research should be deliberately cultivated, this is an opportunity lost.

The central issue between the British government and the House of Lords, however, is on the question whether the research and development enterprise should be politically represented. The House of Lords Select Committee wanted to go back to an arrangement abolished in the 1960s under which the holder of one of the non-executive posts in the British Cabinet would be asked to hold a watching brief for science. Nobody in Britain wants a science minister on the French or German pattern, and indeed there is much to be said for sticking to the present assumption, sanctified by Lord Rothschild in 1971, that the usefulness of public science is enhanced if ministries individually are persuaded to take research and development seriously. Yet as things are, and because of the way that British governments function, the vital task of coordination is bound to be neglected. So it has proved with badgers, acid rain and the dual-support system. The value of a part-time minister is that only such a person can intervene in the British political process at that level at which the doctrine of ministerial independence is subsumed in the more general doctrine of the collective responsibility of cabinet members. The government in its reply to the House of Lords seems deliberately to have missed this point (as also in its rejection of the proposal that a chief scientist should be appointed to the Department of Education and Science with the remark that there are already civil servants to look after the teaching of science in the schools). The manner of its rejection of the proposal is two-fold: first, the

committee-engineering proposed should obviate the need for a nominated minister but, second, there is such a person already in the person of the Prime Minister (who has a degree in chemistry), who has now reaffirmed her interest in science and technology. Should not the House of Commons put her to the test?

Crunch in West Germany

Deutsche Forschungsgemeinschaft may have to break with convention if the squeeze continues.

There is nothing like uncertainty to induce compliance with unpalatable circumstances. This is the simplest explanation for the mood of resignation, not revolt, in which the annual meeting of the Deutsche Forschungsgemeinschaft (DFG) in Bonn last week received the news that, for the first time since the Second World War, its fund (the only fund) for making grants to academic researchers is about to shrink in real terms. (The total budget will increase by four per cent to DM850 million, but inflation is running at six per cent.) The uncertainties that seem to have induced this state of affairs are of two kinds — general concern about the health of an economy which, although still the strongest in Western Europe, is not easily able to hold a candle to Japan, and more immediate anxiety that erosion of the political support for the Bonn coalition government will continue.

But why think of complaining about a reduction of resources that amounts to a mere two per cent? The simple truth is that there are many circumstances in which university research groups will be more seriously affected. In West Germany, the subvention of university research by DFG accounts for a comparatively small part, perhaps only a fifth, of the total cost of university research. The remainder is provided directly by the *Länder* governments, many of which are short of funds because of the recession and are taking the knife to their universities as a consequence. After several years in which university laboratories have been ill-provided with equipment, and with the now high cost of renewal and replacement, it is inevitable that DFG should be spending a growing proportion of its shrinking funds on material that would previously have been provided on the regular budgets of universities. The result is a painful squeeze on the most important of all DFG's functions — short-term support for bright young people with a contribution to make to research. No wonder that Professor Eugen Seibold, the president, was telling DFG last week that it could not stomach another year of this.

Seibold will, however, be lucky if the politicians have the time to listen. Moreover, there is little chance that economic circumstances will have changed sufficiently by the last quarter of this year for the federal government to draft a markedly better budget. So it is high time that some serious thought was given to the simple question whether the present balance between the different sources of support for academic research can possibly be for the best in the best of all possible worlds? There are three sources of support for academic research in West Germany — the *Länder* governments, DFG and the Max-Planck-Gesellschaft. The first two support research *in situ*, the third in constitutionally and sometimes even geographically separate institutes. Even in the abstract, there is a case for holding that DFG should grow at the expense of its two partners in this enterprise. The *Länder* governments are less able to discriminate between the good and the less good projects than they might be, and in any case tend to become committed to people or departments for years on end. And however hard it tries to do otherwise, the Max-Planck-Gesellschaft is capable of setting up academic laboratories that are also ivory towers. The trouble is, of course, that the *Länder* governments are collectively more powerful than even the federal government, while the more or less even balance between DFG and the Max-Planck-Gesellschaft in the past heady decade of prosperity is a mark of able people's mutual respect. What matters about spending money, however, is that it should be spent by those who spend it best. (The parable of the talents refers.) If the next DFG budget also embodies a reduction, Professor Seibold may have to speak up and speak out.

France creates new super-ministry

Chevènement adds industry to research

M. Jean-Pierre Chevènement, formerly French minister of state for research and technology, has risen to new heights in last week's cabinet reshuffle. Far from being affected by his gaffe over the Palestine Liberation Organization leader Yasser Arafat (see *Nature* 1 July, p.2), Chevènement has been promoted to a new super-ministry, the ministry of research and industry.

Because of the recent nationalizations, Chevènement will there control nearly half the productive power of France together with almost all government civil research and development through the staff of the old ministry of research and technology.

Thus Chevènement comes to be what must be the most powerful "minister of the future" in the Western world. Not only has he had a year to develop an energetic

ministry will be called research first and industry second is significant: Chevènement will retain most of his old cabinet of immediate advisers, including his *directeur du cabinet*, M. Louis Gallois, and the man who was to be and will be responsible for seeing into effect the new law on science and technology, M. Roland Morin.

In industry, energy will be one of Chevènement's new portfolios, delegated through the junior minister for energy M. Edmond Hervé. In this field, Chevènement has shown himself catholic: he will consider any form of energy that will free France from dependence on oil. The fast breeder reactor and nuclear power in general are secure with the new minister; and so are biomass and other forms of alternative energy, supported through the recently created Agence Nationale pour la Maitrise de l'Energie.

In the rest of industry, Chevènement's actions are likely to centre on improving

industrial research and development, which has been a weak point in his plan to raise total national spending on research and development to 2.5 per cent of gross national product by 1985. (It stands at 2.0 per cent at present.)

French government spending is already on a par with that of other industrialized countries, critics of the plan have pointed out, and it is industry's effort that has to be raised, not government's. The point was well taken at the ministry of research and technology, and now Chevènement is in a position to do something about it.

The minister is considering fiscal policy (tax incentives) as a means of encouraging private industrial research (despite his early reluctance to use such a "blunt" instrument); and he can take direct action with nationalized industry. Through the nationalized banks, he may also set up sources of venture capital.

Robert Walgate

Hopes for Bell in AT&T case

Washington

The argument over Bell Laboratories' proper role in the soon-to-be unregulated American Telephone and Telegraph Corporation (AT&T) continued last week, as Federal Judge Howard H. Greene held hearings on the government's proposed anti-trust settlement with the company.

AT&T made clear that in agreeing to give up its local telephone companies (the Bell Operating Companies) it felt it was paying a high enough price for the right to enter the unregulated market in computers and data processing. AT&T has so far been barred from this area on the theory that revenues from its regulated telephone monopoly could end up subsidizing computer research and development, giving it an unfair edge in the open market.

Bell's would-be competitors argued that such cross-subsidies could still occur under the settlement, since AT&T would keep its *de facto* monopoly on interstate telephone traffic. Bell Long Lines currently handles more than 96 per cent of this traffic.

The crux of the matter is what role Bell Laboratories will play. At present, 80 per cent of Bell Labs' basic research is supported by the "licence contract fee" that the Bell Operating Companies pay to AT&T. This source will be lost after the settlement.

William Keefauver, vice-president and general counsel of AT&T, says that although this will cause problems, they will be "manageable". He says AT&T intends to maintain Bell Labs as a "first class research outfit" by making up for the lost support with increased contributions from Long Lines (which now supplies the remaining 20 per cent of basic research costs) and with contributions from

American Bell, the new AT&T subsidiary created to go into the computer market. Estimates of the Bell Operating Companies' current contribution to basic research at Bell Labs vary from about \$200 million to \$400 million, depending on how basic research is defined.

Judge Greene is expected to rule quickly. He has a limited range of choices: he can approve, disapprove or suggest changes. Disapproval would mean reopening the case, which has been going on for eight years already. Action in Congress, however, may make the ruling irrelevant. Representative Timothy Wirth (Democrat, Colorado) has introduced legislation that would make the proposed settlement into a law — but go a few steps further. The bill (HR 5158) stipulates that any transfer of funds from Long Lines to Bell Labs must be in the form of a contract for specific research. Any patents arising from such work could be made available to the unregulated portions of the company — American Bell, Western Electric (which manufactures telephone equipment), and AT&T's international division — but only if they pay for it, just as any outside firm would have to do.

AT&T has fought an intensive lobbying campaign against the bill and has spent \$3 million on it. Keefauver argues, however, that the bill would do serious damage to Bell Labs' traditional strength in basic research. He says the requirement that regulated and unregulated research support be separated is unrealistic when basic science is involved: "How do you know where your research is going to go? Electrons and protons don't know whether they're regulated or unregulated."



Chevènement: ambition undimmed

politics of science and technology, enshrined in a law given final approval by the National Assembly last Wednesday, but now he has at his disposal the means to put it into action.

Strangely enough, Chevènement was once firmly against the idea of a joint research/industry ministry. When a previous government set up a ministry of industry and research in 1970, he took the line that research would be submerged and forgotten — which proved to be the case. Now he sees the joint ministry as a "lever" of social change.

All this grand politics has provoked disquiet in the universities, where some fear that even fundamental research will be drawn willy-nilly in Chevènement's politically determined directions. Others, however, hope that Chevènement will now be so concerned with the more immediate politics of industrial affairs that he will let research alone.

Nevertheless, the fact that the new

The subcommittee staff counters that AT&T last autumn said it could live with a provision in the Senate's version of this bill that required not only separate accounting, but an actual split of Bell Labs into regulated and unregulated divisions. Keefauver says that was only because the Senate bill would also have allowed AT&T to hold onto its operating companies.

The Office of Technology Assessment (OTA), which was asked to testify at the House hearings on the bill, has suggested, however, that AT&T's major objections to HR 5158 may not be directly related to Bell Labs. OTA staff point out that the anti-trust settlement itself will have an enormous effect on Bell Labs' basic research mission, if for no other reason than by putting a premium on applied research as AT&T enters the unregulated computer field. By comparison, OTA says, HR 5158 does relatively little.

The bill does, however, make some substantial changes in the anti-trust settlement's treatment of the Bell Operating Companies. It would in particular prevent AT&T from taking over the very lucrative Yellow Pages, the operation of pay phones and the sale of terminal equipment from the operating companies. AT&T would get all of these, along with a large share of the intrastate lines, under the settlement — leaving the newly-independent operating companies with nothing but the right to "market a dial tone" as one critic put it.

The House Energy and Commerce Committee is expected to deal with HR 5158 quickly but the chances of its being passed are only fair.

Stephen Budiansky

Spain for CERN

Spain has rejoined the European Centre for Nuclear Research (CERN) in Geneva, but at a reduced price — less than a third of what it should pay on the CERN formula linking subscription to gross national product.

Starting next January, Spain will pay 2.1 per cent of the £170 million CERN budget (about £3.5 million), rising to its full 7.1 per cent only in 1989. The reduced subscription has been agreed because Spain as yet has too few high-energy physicists to make full use of membership. CERN will allocate the extra cash two ways: 40 per cent to reduce the contributions of other member states and 60 per cent to experiments.

Spain's benefits will be various. The move will help to prove Spain's interest in Europe when it is applying for membership of the European Economic Community. The few Spanish people already at work at CERN will now be able to apply for time on accelerators in their own right, and there may also be contracts to be won for the components of LEP, the large electron-positron collider that will be CERN's next accelerator.

Robert Walgate

French research law

La loi at last

La Loi is the law. Last week the French law for research and technology was finally adopted by the National Assembly in a form much like that originally proposed by the government, despite vehement Senate opposition which put it many weeks behind schedule. Now the race is on to promulgate the various consequent decrees which, for example, will redefine the role of organizations such as the Centre National de la Recherche Scientifique (CNRS).

While the research and industry ministry claims that the law adopted is "90-95 per cent" the same as the original, others point to "substantial" improvements in the protection afforded by the law to basic science. One amendment, for example, mentions the importance of fundamental research to the whole programme.

There is, however, no escaping the fact that the law is basically to do with technology, and with two objectives: to stimulate French effort in the new technologies and to "open up" French science to industry. The key provisions of the law are as follows:

- To raise the government budget for civil research and development by a real 17.8 per cent per year (on the average) to 1985.

- To set up seven "programmes mobilisateurs" — tactical programmes (such as in biotechnology) on which the rest of the law (and funds) will be focused. The list of programmes may be modified each year, but for now will be: the rational use of energy and new energy sources; biotechnology; electronics; research and the Third World; improvement of working conditions; promotion of French as a scientific language; and a programme covering various industrial technologies.

- The creation of regional committees for research and development, consultative to the regional councils, which will have their own budgets and a degree of autonomy to develop a regional science policy.

- The creation of a new legal category of organization, "*établissements publics à caractère scientifique et technologique*", which will be applied to organizations such as CNRS. The formula will give these bodies an explicit duty to attempt to apply their work, to publicize their research and to train and educate researchers.

- A second category of organization — "*groupements d'intérêt public*" — is created to allow bodies such as CNRS for a limited period to form profitable liaisons with industry.

- In a kind of inclusive job description, the "*mission*" of a researcher is defined as the development of knowledge, its transfer to industry and society, its popularization, the education and training of young and old and administration.

- All government researchers will become "*fonctionnaires*", giving them the envied security of civil servants which, hitherto,

only university staffs have enjoyed.

Many in France consider these last two provisions as the most important. One outcome may be to increase the mobility of scientists between research and other jobs. Thus, say the hopeful, university careers may be unblocked while industry may be able to recruit more researchers.

Robert Walgate

European research ministers

Going where?

Brussels

An unexpectedly lively debate between the EEC's research ministers took place last week when they met in Luxembourg to examine the flood of proposals from the European Commission. A distinctly positive response greeted the plans to change the structures of EEC research programmes and to launch the ambitious "Esprit" programme on information technology (see *Nature* 3 June, p.352). More detailed plans are to be prepared on both for the next research council in November this year.

The next stage will be to prepare detailed proposals by 1 January 1982 on the bulk of the Esprit programme, by which time the planned pilot projects should have established the credibility of the concept.

Discussion at the council meeting was also devoted to the Super Sara project at the EEC's Joint Research Centre at Ispra in Italy which will carry out research on nuclear safety. There was some grumbling that it is likely to cost more than at first estimated, but no definite decision was made on this or the ideas put forward for all the joint research centres.

A squabble did develop over the 1982-86 research programme on medicine and public health, for which the Commission had proposed a budget of 20 million units of account (£36 million). Most countries preferred 16.9 million units of account, while the British and West Germans argued in favour of even less, 10 million units. Before there is official agreement, however, the European Parliament will have to be consulted.

Commission officials will be heartened by the seriousness with which the member states are now taking the urgency of starting large-scale cooperation not only on information technologies but in other sectors too. A fully-fledged framework programme on the stimulation of the Community's scientific and technological potential in the 1980s will be ready in November. "A new leaf has been turned in the history of the Community's research strategy. The fragmentary approach which characterized joint Community research and development in the past has been left behind and we are now moving towards objectives which reflect the EEC's real priorities", *Vicomte Etienne Davignon* was reported to have told the ten ministers in Luxembourg.

Jasper Becker

Biological weapons

No NIH ban

Washington

An amendment to the National Institutes of Health (NIH) recombinant DNA guidelines that would have banned the construction of biological weapons by molecular cloning was rejected last week by the Recombinant DNA Advisory Committee (see *Nature* 17 June, p.527).

The committee accepted the assurances of the US Army and the Arms Control and Disarmament Agency that no such work is being conducted and that, in any event, the 1972 Biological Weapons Convention effectively prohibits the construction of biological warfare agents by any means.

After rejecting any change in the guidelines, the committee adopted a much milder resolution that simply advises the director of NIH that the 1972 treaty applies to recombinant DNA research. The treaty forbids the development of biological agents or toxins "of types or in quantities that have no justification for prophylactic, protective or other peaceful purposes".

Meanwhile, both the Office of Management and Budget (OMB) and the Department of the Army have issued official explanations of the US defensive biological warfare research programme. OMB now says it was wrong when it stated last month that the Army had both classified and unclassified budget items in this area. All research on biological weapons defence is in fact in the published budget.

According to the Army statement, this research is limited to two major projects: medical defence, and detection and protection. The medical research, with a budget of \$17 million, is conducted openly at the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland. It focuses on the development of vaccines and treatments for both natural diseases and potential biological warfare agents.

According to Joseph Campbell of OMB, it was a proposed increase in the medical programme that caused concern at OMB earlier this year. Campbell says he wanted to know in particular if a plan to spend \$75–100 million over the next ten years for the development of an anthrax vaccine made sense.

The detection research is being done at Aberdeen Proving Ground in Maryland. According to Thomas Dashiell of the office of the Secretary of Defense, its budget is \$3.8 million this year. A chemiluminescent detector sensitive to bacteria and naked viruses has been developed. The project itself is unclassified, although information on the detector's sensitivity is secret. Research on protective clothing and decontamination is all being done under the chemical weapons research budget.

Dashiell says the remainder of the biological defence budget — \$200,000 — goes to a small "technology watch" programme

run by the "intelligence community", and which is classified. The aim is keep an eye on developments throughout the world that might affect the vulnerability of US and NATO forces to biological attack.

Dashiell was also able to confirm that a request to the National Academy of Sciences for studies on chemical and biological weapons issues originated from the Under Secretary of the Army. The academy's assembly of life sciences is now considering a specific request for a literature search on mycotoxins.

Stephen Budiansky

Deep sea drilling

Soviets out

Washington

The Soviet Union will no longer participate in the US International Deep Sea Drilling Program. The bilateral US-Soviet agreement under which it had contributed scientists and \$2 million a year for the past nine years has lapsed, and the White House has ordered that it should not be renewed.

The termination of the accord is part of a general withering of US-Soviet scientific ties that started with the Soviet invasion of Afghanistan in December 1980 and intensified after the imposition of martial law in Poland last December. At that time, President Reagan ordered that the US-Soviet bilateral agreements then in force could continue, but should be terminated as each came up for renewal.

Although the deep-sea drilling programme agreement is not to be continued, the US-Soviet oceans accord, of which the deep sea drilling agreement is part, is still in force. The space agreement lapsed in May, the energy agreement in June and the science and technology agreement will lapse this month. The oceans agreement continues because it was renewed last December, before the Polish crackdown.

Several other agreements continue: in transportation, housing, atomic energy and agriculture research. But they are "pretty moribund", says one White House science official. The most active, perhaps, are exchanges relating to fusion energy and high-energy physics — areas of traditional US scientific cooperation abroad. These are part of the atomic energy accord. Several agreements come up for renewal in the autumn, which will be the President's next chance to signal his view of the state of US-Soviet relations.

The departure of the Soviet Union from the ocean drilling programme will probably be more than made good by the participation of other countries. At a meeting in Washington last month, countries such as Australia, Canada, New Zealand and Switzerland expressed interest in the Advanced Ocean Drilling Project.

The plan is to use the 52,000 ton *Glomar Explorer*, originally built by the industrialist the late Mr Howard Hughes to

recover a Soviet submarine from the Pacific, as a replacement for the much smaller *Glomar Challenger*. The National Science Foundation thinks that *Explorer* could be ready as early as 1985, provided that sufficient support (including that from overseas) materializes. Contributions from outside the United States have met a third of the cost of the *Glomar Challenger* programme, but this proportion may have to change now that the oil industry has pulled out of a joint venture in *Explorer*.

Deborah Shapley

Rayner on government research

Sceptics abound

Sir Derek Rayner's proposals last week for reducing peripheral waste in British government research establishments (*Nature* 1 July, p.3) have encountered a sceptical response. Some staff representatives say that the proposals are based on scanty and misleading analyses. Some of the central government departments, which will have the final say over which proposals to accept, are said to share that view.

Rayner's proposals were based on studies in 19 laboratories looking for ways of cutting support services without jeopardizing research.

Most of the response to the proposals so far has come from the Institution of Professional Civil Servants (IPCS), whose scientific staff members are not directly affected. Unions representing cleaners, clerical, engineering and technical staff, among whom Rayner suggests savings of 19 per cent would be possible, will take longer to reply, partly because there are so many of them and partly because few of them are used to dealing with Civil Service problems.

IPCS says that it would welcome greater efficiency in the services available to its members and that few scientific staff would shun the increased management responsibilities the Rayner proposals would give them. But many of the proposals, it is claimed, either imply a change of government policy towards research or will not achieve the estimated savings.

Thus, IPCS says, suggestions that laboratories charge economic rates for information supplied conflict with policies for disseminating much government research as widely as possible. And the recommendation to contract out for as many services as possible overlooks the need for highly specialized services in government laboratories.

The proposals for shedding laboratory land and buildings, however, seem to have aroused the greatest scepticism. Thus critics say that moving the Princes Risborough outstation of the Building Research Establishment to the main site at Garston would not save the £343,000 mentioned by Rayner. According to

members of the staff, the true costs of moving have not been deducted from the savings and they doubt that the vacated site could be sold at market value during a recession.

Similarly, staff at the National Physical Laboratory claim that the proposal to close 200,000 square feet of buildings on the main site makes no economic sense. Some of the buildings recommended for closure are said to contain equipment such as standard force measurement machines and a vibration-free table whose removal would cost far more than the proposed savings.

Judy Redfearn

Levich in New York

Down to work

New York

The fourth "Levich" conference held last month in New York was more like a routine scientific conference than a human rights protest. Organized by the New York Academy of Sciences and the City College of the City of New York, the Fourth International Conference on Physico-Chemical Hydrodynamics squeezed its concern for scientists' freedom in Eastern Europe into only a short tea-break. Professor Benjamin Levich, of the Weizmann Institute in Israel and simultaneously Einstein professor of physics at City College, participated as honorary chairman.

Five years ago at the first conference, Professor Levich was still in Moscow, a "refusnik" refused a visa to emigrate to Israel but also prevented from continuing his scientific work. Deprived of the sixtieth birthday conference normally accorded to Corresponding Members of the Soviet Academy of Science, Professor Levich was instead honoured with a conference organized by his colleagues in the West. The Soviet scientific establishment castigated this as an attempt to "set the scientific world in the West against the Soviet Union" — a charge strongly denied by Sir Derek Barton and Professor Brian Spalding, who stressed the "high importance" of physico-chemical hydrodynamics and of Levich's work in the field.

Nevertheless, the fact that the conference took place without the guest of honour inevitably publicized — and was intended to publicize — Levich's plight. When, the following year, a similar "Levich birthday" conference was convened in Washington DC, presumably in honour of his sixty-first birthday, the scientific purpose of the conference was again coupled with the desire of Levich's colleagues to win him the right to emigrate.

By the third conference (Madrid 1980), this aim had been achieved; Professor Levich had been in the West for more than two years and could preside in person. At the fourth conference human rights were referred to only in passing — in a review of the current situation presented by the

Committee of Concerned Scientists and in an evening entertainment by a drama workshop from City College.

The conference, with its papers ranging from Czochralski crystal growth to three-phase coal slurries and from the haemodynamics of arterial flow with stenosis to two-dimensional flame propagation, honoured Levich rather by implication, indicating the wide ramifications of the discipline he helped to develop. Invited participants from the Soviet Union were unable to attend.

Vera Rich

Acid rain

UK unrepentant

Stockholm

The Swedish government scored a modest success last week with its *ad hoc* meeting of the signatories to the 1979 Geneva Convention on long-range transboundary air pollution. The ministerial meeting accepted an expert report on the state of knowledge on acid rain, produced at a meeting the previous week, and it now seems likely that the convention will come into force by the end of the year.

Under the Geneva Convention, polluting countries must reduce sulphur emission, and the expert report effectively removed many of the objections that have been raised. There is no longer any doubt that sulphur dioxide and nitrogen oxides are responsible for the damage done to 20,000 Scandinavian lakes and a million hectares of central European forests.

Even so, Sweden's attempt to revive the "spirit of 72" when the UN Conference on the Environment was held in Stockholm was a flop. Some of the worst polluters, such as the United Kingdom, France and the United States, were complacent. Britain's Giles Shaw, Under-Secretary of State for the Environment, admitted the United Kingdom's burden of responsibility as Western Europe's biggest source of sulphur dioxide emission, but said that considerable strides had been made since 1972. Britain claims to have reduced sulphur dioxide emission by more than 20 per cent but mainly as a consequence of economic recession, the use of natural gas and low-sulphur North Sea oil and a greater use of coal.

The real surprise at the conference, however, was the change of heart by West Germany, where Chancellor Helmut Schmidt is anxious to win back the ecological vote after his party's near-defeat in the Hamburg elections. Another factor is the research carried out by Professor Bernhard Ulrich of the University of Göttingen which shows that 40 per cent of German forests have started to die, almost certainly as a result of air pollution. The expert report, however, considered as inconclusive the evidence that acid rain directly affects tree growth.

Scandinavian forests are not so far

affected, principally because of the lower concentrations of sulphur dioxide and nitrogen oxides there. An ability to predict the speed at which acid rain will affect soils outside Scandinavia or the forests in that region, as well as the exact relationships between emissions and long-range precipitation, is crucial if the Scandinavians are to persuade other countries to spend money on pollution control. But the experts say that more research will be required before this can be done.

Some action at least is being taken. The Netherlands proposed reducing yearly per capita emissions of sulphur dioxide to 35 kilogrammes (the figure for the United Kingdom is about 88 kilogrammes) and nitrogen oxide emissions from 40 to 20 kilogrammes, which should halve the average wet deposition of sulphur in Europe. Although this proposal has not yet been accepted, the conference agreed that "even if deposition remains stable, deterioration of soil and water will continue and may increase unless additional control measures are implemented".

Jasper Becker

US 1983 budget

Tighter still

Washington

The 30 per cent of US researchers who depend on federal funds are now a little closer to knowing how much money they will have when the new fiscal year begins on 1 October. The House of Representatives and the Senate have finally approved a budget resolution setting targets for government appropriations, that is, how much money may actually be spent in the 12 months starting in October. The Appropriations Committees of the House and Senate, each with 13 subcommittees, will now start working out individual spending figures.

A parallel but related process in Congress determines authorizations — the upper limits of what can be spent as well as approval for future years' programmes. Each process can modify the original budget request by the President but he has a veto. President Reagan has already vetoed a supplemental appropriation that contained, among other things, money for student loans, because it also included a housing measure he disliked. (Another supplemental appropriation, with the loans but without the housing, is expected to be passed soon.)

But last month's resolution may not put an end to the budget controversy that has dogged the Administration and Congress for most of the year, for the figures are somewhat higher than the President requested in domestic programmes, and somewhat lower for defence. If the appropriations committees agree these figures, the President could veto their measures. Even Washington has been bemused by the budget high jinks this year.

This may have become a perennial problem. Two weeks ago, Michael L. Telson, an analyst with the House Budget Committee, warned the assembled administrators that the fight over the budget resolution was prophetic of what will happen in coming years.

He was speaking at an annual discussion of the federal research and development budget sponsored by the American Association for the Advancement of Science and based on a report* it issues each year. Telson said that so long as the Administration sticks to high defence spending and no major new tax revenue and does not lower the entitlements part of the budget (payments to individuals), the discretionary part of the civilian budget will become the scene of intense political competition. "Don't be surprised if you have trouble in the appropriations process that you never had before", Telson warned the group. "And don't take it personally."

Telson presented a chart from the President's budget request in which civilian research and development is included in the "all other" column. It shows how much less money there will be overall in the fiscal year 1983 than even in fiscal 1982.

David A. Shirley, director of the Lawrence Berkeley Laboratory (LBL) in California, gave the meeting an idea of what the recent budget shifts mean at the working level. LBL has no major weapons programmes or nuclear programmes and so has been particularly vulnerable to shifts in the civilian budget. When the President released the budget for the fiscal year 1982 in March 1981, the laboratory realized it

would have to cut personnel by 10 per cent on 1 October. On 24 September, however, the Reagan Administration issued a revised budget for fiscal year 1982 which required a further cut of 300 employees, bringing the combined cut to 19 per cent. Seventy per cent of these cuts were made in basic research, Shirley said.

Now, Shirley said, there is a third cut coming for fiscal year 1983 in October, but he does not know its size because Congress and the Administration have not agreed on a budget. Legally, however, as director of the laboratory, he must give employees 90 days' notice of termination. He can make a guess now (something even soothsayers in Washington are not doing) or he can have the "full" complement of employees working on 1 October, and then make larger cuts later.

Meanwhile, Dr George A. Keyworth, the President's science adviser, complained of the hue and cry scientists had raised about changes that had been proposed — which he described as a quest for priorities — in the year since he took office. On the whole, however, Keyworth said the scientists, meeting with him privately in small groups of "20 or 30 a day", had been constructive, and anxious to help him set priorities. He boasted that the President's budget had done rather well by science — a conclusion the AAAS report supported — and took recent increases of 16 per cent in research and development spending by industry as evidence that US science is on the road to recovery (although others have attributed much of it to changed accounting due to new tax concessions). Having delivered his talk, and answered questions, he was out of the door before the moderator of the meeting had finished thanking him.

Deborah Shapley

*W.H. Shapley, A.H. Teich & J. Weinberg: *Research and Development, AAAS Report VII*. (American Association for the Advancement of Science, Washington, DC).

Fancy technique

St Louis

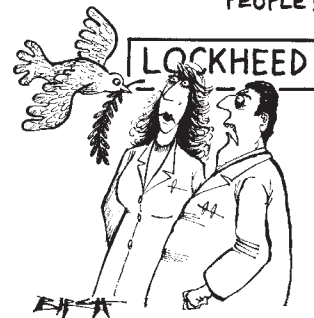
From an industrial giant in the heart of California's "Silicon Valley" that specializes in conquering space in a minimum of time comes the latest in rapid communication — the homing pigeon.

At Lockheed Missiles and Space Company, a division of Lockheed Corporation, draughtsmen have gone beyond pencils and T-squares to perform their intricate design work on a computer's video screen.

A courier had to spend 1½ hours travelling over congested highways and winding mountain roads to carry print-outs from the Sunnyvale computer, 20 miles away, back to the designers, and designs done one day did not arrive until mid-afternoon on the following day.

That's where the homing pigeon stepped, or flew, in. The idea arose when someone heard about a hospital in England using the birds to transport blood samples. The company directed a

"THAT'S THE THIRD THIS WEEK
TO BE GOT AT BY THE FREEZE
PEOPLE!"



research chemist, Werner Deeg, to investigate. Deeg built a pigeon loft, and started with eight pigeons donated by local pigeon fanciers. He now thinks of pigeons as his hobby, and tends them in his lunch hour.

The pigeon courier service has been running since mid-December. A pigeon gets a lift down the mountain to Sunnyvale every afternoon with the regular courier. At the end of a day, a microfilm copy is made of the print-outs and the following morning the pigeon heads for home with the microfilm strapped to its leg. It's just a 20 minute flight as the pigeon flies.

The weather is the only problem said Deeg. "But, even so, we've been able to fly 85 per cent of the time." Why doesn't Lockheed have electronically-operated printers to transmit the data? It does — but at \$10 a print, that system is used only as a back-up. The pigeon can carry a day's work, 30 or 40 blueprints, at a cost of about \$1.50.

"They live 12–15 years unless they're eaten by a hawk or fly into electric wire", Deeg said. He's proud of their record so far: "We've yet to lose a pigeon or a microfilm."

Karen Freeman

Universities outflanked by business lobby

Washington

Congress gave final approval last week to a plan that will set aside 1.25 per cent of federal research funds for small businesses. The legislation, almost certain to be signed into law by President Reagan, was strongly opposed by universities, fearing reduced spending on basic research.

The universities, however, were no match for small business, which has a powerful position in Washington. The House of Representatives passed the measure by an overwhelming vote of 353–57 and it was quickly approved by Senate (which last December had passed its own, similar version, by a 90–0 vote).

Any federal agency having a research budget of more than \$100 million will be affected by the new measure. This includes the National Aeronautics and Space Administration, the National Institutes of Health, the Environmental Protection Agency, the Veterans' Administration, the Nuclear Regulatory Commission and the departments of defence, energy, agriculture, transportation, interior and

commerce. After a three-year phase-in, the sums set aside will reach 1.25 per cent of each of these agencies' extramural research budgets (one-and-a-quarter per cent of the 1982 budgets would total \$377 million).

The chief concern of the universities is that although the percentage cut appears small, its effect on basic research funds will be greatly magnified. A spokesman for the Association of American Universities called it "very unfortunate". A large proportion of spending on current research goes into fixed costs and previous commitments to multi-year projects, he said. What is left is the more vulnerable support for new, basic research projects.

A deeper worry is that since small businesses — defined as firms with fewer than 500 employees — will still be able to compete for funds under the regular programmes, the fund set aside will go to proposals that are unsuccessful in the general competition for funds. The bill's backers claim that peer-review of set-aside fund proposals will eliminate that problem.

Stephen Budiansky

CORRESPONDENCE

Cashing in

SIR — In *Nature* of 28 May (p.260), De Witt Stetten Jr writes concerning the release of the gene of Veniality and the epidemic produced thereby. Enjoying as he does a healthy income as an author of one of the best biochemical textbooks around, but being precluded from participating in the gene technology largesse by virtue of his employment as a civil servant, Dr Stetten makes a witty but not inaccurate statement as to the consequences of the new commercial value to gene technology in terms of suppressing the totally free exchange of information which was, for many years, characteristic of biomedical research. However, in order to establish a risk/benefit ratio, someplace along the line the benefit must also be mentioned; it is not just that the socioeconomic status of the upwardly mobile scientist in gene technology is improved (important though that may be!), but there is reason to expect a profound acceleration of scientific development for human use to occur.

A number of foundations have been predicated on the clear and demonstrable proposition that the rate of transformation and transport of information from the laboratory bench to the clinical bedside is extremely slow and tends to fall outside the purview of the grant support system developed over the years by the National Institutes of Health. Venture-capital supported gene technology is certainly one example (together with hybridoma technology and other forms of research and development partnerships) wherein a much more rapid translation of laboratory discoveries to the benefit of human consumers can be accomplished. It is in the context of this benefit that the risk to scientific communication should be evaluated. I, for one, would rather see the scientist "make a buck" than be excluded from that process by non-scientific entrepreneurs!

JOHN C. HOUCK

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Known quantity

SIR — Semi-log notation, as advocated by J.A. Nicoll (*Nature* 10 June, p.450), is already known, see Danloux-Dumesnils, M., *The Metric System*, 152 (Athlone, London, 1969).

OLIVER MILBURN

Leeds, UK

Earth science in Ovid

SIR — Two passages in Ovid's *Metamorphoses* should be of interest to geologists for the way they illustrate some early ideas that without too much exaggeration could be epirogenic, geomorphic and vulcanologic. The first passage occurs in a long philosophical peroration delivered by Euphorbus of Samos; the second in a song by one of the Aonian sisters, all of whom were too vainglorious for their own good.

Euphorbus declaims:

Nothing, I feel sure, lasts long under the same appearance . . . thus often has the condition of places changed. I myself have seen what once was solid land changed into sea; and again I have seen land made from the sea. Sea shells have been seen lying far from the ocean, and an ancient anchor has been found on a mountain top. What was

once a level plain, down-flowing waters have made into a valley; and hills by the force of floods have been washed into the sea . . . Zancle also is said to have been a part of Italy until the sea washed away their common boundary and thrust back the land by the intervening waters. If you seek for Helice and Buris, once cities of Achaia, you will find them beneath the waves; and the sailors still show you the sloping cities with their buried walls.

Callopie, the Aonian — with harp and song to Ceres?

The huge island of Sicily had been heaped upon the body of the giant, and with its vast weight was resting on Typhoeus . . . He struggles indeed, and strives often to rise again . . . and Aetna's weight is on his head. Flung on his back beneath this mountain, the fierce Typhoeus spouts forth ashes and vomits flames from his mouth. Often he puts forth all his strength to push off the weight of the Earth and to roll the cities and the great mountains from his body: then the Earth quakes, and even the king of the silent land is afraid lest the crust of the Earth split open in wide seams . . .

M. KAMEN-KAYE

Cambridge,
Massachusetts, USA

1. Miller, F.J. (transl.) *Ovid in six volumes, III Metamorphoses* 3rd edn. I, 263 (Harvard University Press, 1977).
2. *ibid.* II, 383; 385.

Vietnam now

SIR — Large numbers of individuals in Indo-China, both military and civilian, were inadvertently exposed to dioxin (TCDD; 2,3,7,8-tetrachlorodibenzo-para-dioxin) as a result of US actions during the Second Indo-China War. This continues to be a matter of grave concern owing to the toxic, teratogenic, carcinogenic, and mutagenic properties of this substance. I provide the following information on dioxin levels in Vietnam owing to the widely divergent values that keep appearing especially in the popular and semi-popular literature, often incorrectly attributed to me.

Dioxin was disseminated as an impurity of the 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) component of the now notorious anti-plant chemical weapon code-named Agent Orange; and also of its much more modestly used close relatives (Agent Orange II, Pink, Purple, and Green), all subsumed here under Agent Orange. The Agent Orange was dispensed by the United States between 1962 and 1970 (primarily during 1966–70) in attacking inland and coastal forests and some crop lands. Although Kampuchea, Laos³, and North Vietnam were all thus attacked, it was South Vietnam that was the major recipient⁴.

Approximately 44,300 m³ (57.0 million kg) of Agent Orange was expended by the United States during the war, containing about 24.1 million kg of 2,4,5-T *per se* (ref. 1 p.26). It is difficult to make a reliable estimate of the actual amounts of dioxin contained in the Agent Orange used in Indo-China owing to the untimely destruction by the US Air Force and the Dow Chemical Company of the reference samples of most of the lots used. An estimate based on Dow determinations released by the US Air Force in 1974 is that the Agent Orange

had an average dioxin content of 2.5 g m⁻³, or 1.9 mg kg⁻¹ (ref. 1 pp.44–45). This suggests that a total of at least 110 kg of dioxin was disseminated. Some new information released by the US Air Force in 1980 indicates that the total was somewhat higher, perhaps 170 kg (ref. 5: p.368). As dubitable as these Dow/Air Force data are, I am aware of no others from which to make better estimates. It should also be noted that the total amount of dioxin in Vietnam, whatever it was, was probably augmented to a slight extent as a result of the burning of wood that had been sprayed with 2,4,5-T.

It appears that approximately half the amount of aerially applied dioxin decomposes within a few days, with the remainder becoming more permanently incorporated into the soil and biota. Here it can work its way up food chains, including those culminating in humans, becoming concentrated in the process. The dioxin thus incorporated into the ecosystem appears to disappear following first-order kinetics, having an environmental half life of perhaps 3.5 years (refs. 4,5). In the case of Vietnam, if one makes the simplifying assumptions that the amount of dioxin introduced was, indeed, 170 kg and that it had all been introduced in 1968, then perhaps 8 kg remained in 1980, 3 kg will be present in 1985, and 1 kg in 1990. Its legacy, on the other hand, could persist far longer.

ARTHUR H. WESTING

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Amherst, Massachusetts, USA

1. Westing, A. H. *Ecological Consequences of the Second IndoChina War*. (Almqvist and Wiksell, Stockholm, 1976.)
2. Westing, A. H. in *Harvest of Death*, 177–205 (Macmillan Free Press, New York, 1982).
3. Westing, A. H. *Nature* 294, 606 (1981).
4. Westing, A. H. *Ecol. Bull.* 27, 285–294 (1978)
5. Westing, A. H. *SIPRI Yearbook* (1982).

Too many drugs?

SIR — In *Nature* of 1 April, M. Weatherall¹ reviews the reasons for the fall off in the number of newly introduced drugs using the now familiar arguments of restrictive legislation with its inappropriate toxicity screening procedures and the need to explain to the public the problems involved in solving the risk-to-benefit equation, recently widely discussed both nationally and multinationally^{2,4}.

The pharmaceutical industry accepts⁵ that its future contribution to treating the generally recognized pattern of Western diseases will be minimal, a view substantiated by the US Senate report⁶ linking six of the ten major diseases to dietary imbalance, epidemiological evidence⁷ that repeatedly points to ways of preventing some important cancers, obesity and heart disease, and Hellman's assertion that there will be no magic bullet in the chemotherapy of cancers⁸. In spite of this last statement recent proposals have been placed before the Committee for the Safety of Medicines to introduce a special classification for new experimental molecules which may be potential anticancer drugs. These proposals, if accepted, will allow the clinical trial of molecules, designed using current biochemical knowledge and that possess, theoretically at least, the possibility of interfering with cancer

Continued on page 210

NEWS AND VIEWS

Big-bang reproduction and ageing in male marsupial mice

from Jared M. Diamond

IN 1966 Woolley¹ reported the first mammalian example of big-bang reproduction (semelparity) — a life cycle pattern in which an individual engages in just one intense bout of reproductive activity during its lifetime and dies soon thereafter. Recent papers²⁻⁴ add to earlier information⁵⁻¹³ on this remarkable mammalian case, which is as challenging and significant to developmental biologists and students of ageing as it is to population biologists.

The phenomenon involves at least six Australian species of small insectivorous marsupials of genus *Antechinus*, popularly known as marsupial mice. In *A. stuartii* of south-east Australia, for example, juvenile males disperse from the maternal nest in May at the age of 8 months, establish territories and become sexually mature. In August, the late austral winter, females come into oestrus and mate in a brief but hectic period of male–female copulatory activity and male–male fighting. Within the next two weeks, the males rapidly lose weight and become ill, their sexual organs regress (see the figure) and by the end of August all the males are dead. The cycle is synchronous for all members of the population, which therefore now consists only of pregnant females and no free-living males; the sole males are embryos *in utero*. All births in the population occur within a two-week period in September, and weaning is in December. Juveniles of both sexes begin making forays outside the nest in January, and territory formation starts again in May. As no male survives beyond 11.5 months of age and the population is synchronized, there is no temporal overlap between successive male cohorts *ex utero*. Some, but not many, females survive to breed a second year. However, at least one related species, *A. minimus*, has big-bang females as well as big-bang males: all the females die after weaning their litter.

This bizarre history poses two distinct types of problem. What is it about the environment and biology of these marsupials that led to the evolution of big-bang reproduction? What are the proximate causes of male senescence? Does it result from external forces or is it an internally

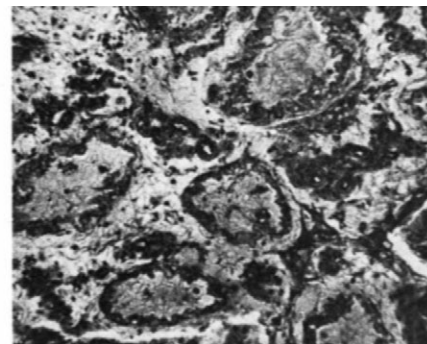
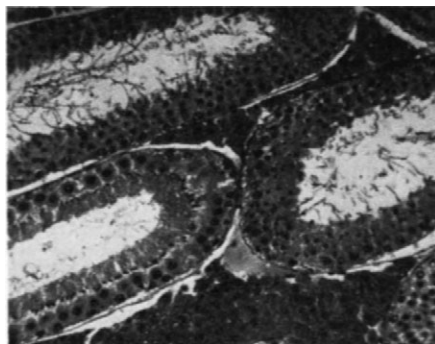
preprogrammed self-destruction?

Consider first the evolutionary problem. We humans take iteroparity (reproduction several times in an individual's lifetime) for granted, because it holds for us and almost all other vertebrates. However, semelparity (reproduction once in a lifetime) is widespread among bacteria, invertebrates and plants. Examples include annual plants, plus species that breed once and then die at an age of a few years or decades: century plants (*Agave*), periodic cicadas, some bamboos and palms, and a few cold-blooded vertebrates (lamprey, Pacific salmon, freshwater eels). Several authors¹⁴⁻¹⁶ have analysed the evolutionary trade-offs between semelparity and iteroparity. Until the recent discoveries among marsupial mice, semelparity was unknown and unexpected among warm-blooded vertebrates for obvious reasons: their relatively low fecundity, high investment in parental care and long lifetimes select for spreading the risk of leaving progeny over multiple reproductive efforts. Why should an individual mammal stake everything on a single bout of big-bang reproduction? Even more surprising, why should all individuals in a population do so synchronously?

For some marsupial mice four factors have been suggested as making this seemingly risky strategy the best one^{12,13}. First, the length of time required for gestation plus weaning is longer in small marsupials,

especially the Dasyuridae (the family to which *Antechinus* belongs), than in similar-sized eutherians (= placentals). For marsupial 'mice' this time is four months, triple that for similar-sized eutherian mice. Thus, more than two litters per year would be physiologically impossible even if sufficient food were always available. Second, big-bang *Antechinus* species live in areas of Australia with seasonal rainfall and temperature, which control the abundance of insects on which they feed. Lactation and peak growth rates of young are timed to occur during the wet spring months, when insect food is maximal. Lack of food in the subsequent dry months would condemn a second litter to failure. Further pressure for synchronous breeding comes from interspecific competition: among sympatric pairs of *Antechinus* species (*A. bellus* and *A. bilarni*, *A. swainsoni* and *A. stuartii*, *A. flavipes* and *A. stuartii*), the two species have their cycles timed 6 weeks apart, thereby reducing simultaneous demands on food. Third, the timing of rainfall and temperature is predictable in areas with big-bang populations. Thus, the risk of losing all the young due to unfavourable weather during the months of lactation is minimal. *Antechinus* species that live in the unpredictable desert environments of Australia's interior, or in the less seasonal rain forests of New Guinea (*A. melanurus*, *A. naso*; *A. wilhelminae*?), do not use a

Left: testis of the marsupial mouse *Antechinus bilarni* at the height of sexual activity in May–July. $\times 83$. The seminiferous tubules are lined by germinal epithelium with abundant spermatocytes and the lumina are wide open. Masses of interstitial cells are visible between the tubules. Right: testis of *A. bilarni* at post-breeding stage of sexual regression in September. $\times 83$. The seminiferous tubules have shrunk in diameter and been reduced to a basement membrane, the germinal epithelium having disappeared. Only small clusters of interstitial cells remain. Connective tissue fills the tubule lumina as well as the interstitial areas. (Figures from ref.3, kindly provided by Dr J. Mary Taylor.)



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big-bang strategy but are iteroparous. Fourth, like other small mammals, marsupial mice have short life expectancies in the wild.

Thus, big-bang marsupial mice are limited by the combination of a seasonal environment and their own physiology to one litter per year born in late winter or early spring. They would be unlikely to survive until the same season the following year. The best strategy is therefore to stake everything on one all-out effort, and the predictable seasons make this a safe strategy. Breeding males indulge in a lethal suite of activities that leave little time for feeding: wandering in search of females, fighting other males and exhausting copulations lasting 5–12 h with each female and repeated daily for up to 11 days. Since a few dominant males may succeed in fathering most of the population's offspring, it pays males to maximize their efforts. By dying promptly after mating, the large aggressive males (nearly double the size of females in big-bang species) remove themselves as useless competitors for food at a time when the females must nurse a litter whose combined weight is up to triple the female's weight. This male altruism permits the females of big-bang *Antechinus* species to rear generally larger litters than females of related semelparous marsupials.

These environmental considerations suggest that further mammalian examples of semelparity should be sought among other small marsupials in predictably seasonal environments and among small eutherians of temperate-zone mountain tops and Arctic habitats with a brief growing season¹³. For example, the shrew *Sorex vagans* is nearly semelparous at altitudes of 10,000 feet in mountains of western North America. The smallest primates, such as mouse lemurs (*Microcebus*) and pygmy marmosets (*Cebuella*), are speculative candidates for semelparity.

To developmental biologists, the interest in big-bang male marsupial mice lies in their synchronized rapid progress from peak condition through senescence to death during the last few weeks of their 11.5 month lifespan. In these weeks, sperm disappear, the prostate gland and Cowper's gland regress, the penis becomes flaccid and the testis is invaded by connective tissue until it is scarcely recognizable as

a testis in light microscopic sections. The males have negative nitrogen balance, lose weight and their basal metabolic rate declines. They are afflicted with hypertrophied adrenals, hepatic necrosis, infection, gastric and duodenal haemorrhage, and anaemia. Low levels of circulating immunoglobulins corresponding to IgG and IgA suggest that infection results from suppression of the immune system. The immunosuppression and some of the other changes may in turn result from increased levels of total plasma corticosteroids and decreased corticosteroid-binding capacity in plasma, hence a marked rise in free plasma corticosteroids^{8,12}. Corticosteroid injections into captive males to yield levels found in post-breeding wild males lead to infections, decreased IgG and IgA levels as in the wild, and death within a few weeks in about half the animals.

Was this lethal stress syndrome a result of the exhausting forays, fights, copulations and lack of time for feeding, or due to a predetermined endocrinological self-destruction? Both explanations apparently contribute. Males captured during the breeding season and thereafter kept isolated in laboratory cages do not necessarily die at the same time as wild members of their cohort but survive up to 2 years longer, depending on their condition when captured. Thus, the external stresses of breeding clearly precipitate death. However, several observations also

demonstrate an annual metabolic cycle independent of external stress. Captive males in isolation exhibit the same weight loss, decrease in basal metabolic rate and regression of sex organs in the late winter as wild males. The captives remain sterile thereafter and do not regain sexual function as their second winter approaches. If first-year males are captured and castrated, they undergo no late-winter decline in weight and basal metabolic rate.

Life cycles of humans and of big-bang *Antechinus* species pose similar questions of ageing, but on a different time scale. Big-bang *Antechinus* males become sterile at 11 months and die 'naturally' at 11.5 months, if they have not died earlier from accident or disease. Female *A. minimus* also die 'naturally', but at about 1.5 years. Female humans become sterile at 40–50 years, and humans of both sexes die 'naturally' at three score years and ten, give or take some. Why do human females not remain fertile until death, and why do human males not become sterile before death, and why do we die naturally at 70 rather than at 40 or at 140? Marsupial mice, with their small size, ease of maintenance in the laboratory, rigidly synchronized cycle and representation of both big-bang and iteroparous strategies in different species or in opposite sexes of the same species, may offer convenient animal models to biologists fascinated by these universal puzzles of ageing. □

Meteorite falls

from David W. Hughes

METEORITES are falling to Earth all the time. But do they hit some places more often than others? And do we get more in the daytime than at night and in summer as opposed to winter?

These questions are difficult to answer because what is detected depends on the distribution of observers (see Hughes *Meteoritics* 16, 269; 1981). The scattering of the sites on the globe at which meteorites have been recovered, after being seen falling through the atmosphere, bears a striking similarity to the distribution of regions of high population, intensive agriculture and scientific sophistication.

There are over 650 meteorites whose time and place of fall are well known and this number is increasing at the rate of about 60 per decade. About 55 per cent of these falls are recorded between latitudes 35°N and 55°N, echoing the distribution of population. The number falling as a function of local time of day shows a pronounced minimum between 0000 and 0500 hours. However, it is not just that more people are up and about during the daytime than at night and therefore more

meteorite falls are observed then. Even though the fireball, produced when part of the meteorite burns up as it is slowed down in our atmosphere, can be seen more easily at night, the chance of finding a meteorite is greater during the day.

Over the year it is found that more meteorites are observed to fall, per month, between April and October than between November and March. Again, although more people are outside in the spring and summer in the Northern Hemisphere meteorites can often be more easily recovered on frozen or snow-covered ground.

Ian Halliday and Arthur A. Griffin of the Herzberg Institute of Astrophysics, Ottawa, Canada have now found a way around these difficulties of observational bias and their conclusions are published in a recent edition of *Meteoritics* (17, 31; 1982).

Networks of cameras have been

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photographing the clear night sky for about two decades now. The Meteorite Observation and Recovery Project set up by the National Research Council of Canada in the Canadian Prairies is one example of such a network. Careful analysis of the data gathered enables the orbital characteristics of the meteorite precursors to be recognized. It seems that a typical precursor orbit is direct (moving in the same sense as the planets), has a low inclination to the plane of the Earth's orbit, has aphelion about 3.3 AU from the Sun, in the asteroid belt, and perihelion at about 0.9 AU. Halliday and Griffin have taken a further step and specified a distribution of orbits with varying inclinations, semi-major axes and perihelion distances that adequately represent all the precursor orbits. Given this distribution the expected variation of meteorite fall sites with latitude can be predicted, as can the meteorite fall statistics as a function of time of day and season of year.

One thing that must be taken into account is that the Earth's atmosphere acts as a filter. Interplanetary objects, gravitationally bound to the Sun, can hit the Earth at velocities of between 11 and 74 km s⁻¹ (the former objects are 'overtaking' us, the latter suffering a 'head-on collision'). Objects with geocentric velocities in excess of about 22 km s⁻¹ do not survive the deceleration in the atmosphere and thus cannot produce meteorites.

Halliday and Griffin find that the Earth's gravitational field has an important effect on the orbit of the incoming meteorite precursor. This field tends to spread the fall sites over the globe. There is only a modest decline in the predicted meteorite fall rate as one moves from the equator to the pole, the polar rate being about 85 per cent of the equatorial rate.

Plotting the influx rate as a function of local time of day shows that there is a pronounced minimum at 0600 and a very broad maximum between noon and midnight. The variation becomes less as one moves from the equator to the pole but, taking latitude 52° as an example, the maximum flux is about twice the minimum here. Just considering daylight falls (0600 to 1800 h, a period during which most people are awake and can reasonably be expected to observe a meteorite fall), the authors calculate that 62 per cent of the falls should occur after 1200 h. This is in reasonable agreement with the observed ratio of afternoon to morning falls.

Finally, turning to time of year it seems that the influx rate for both hemispheres is highest in springtime. The variation is hardly noticeable at the equator but, again at latitude 52°, the autumnal influx is only 70 per cent of the springtime value. So the sowers are favoured over the harvesters if, like me, you regard meteorites falling out of the sky as something to be thankful for.

Transport of sugars during glycoprotein synthesis

from Martin D. Snider

RECENT studies have made it reasonably clear how the eukaryotic cell goes about the complex task of assembling those secreted and cell-surface glycoproteins that carry an asparagine-linked oligosaccharide. The first stage involves the assembly of a fourteen-sugar oligosaccharide on the polyisoprenoid lipid carrier, dolichol pyrophosphate. This oligosaccharide-lipid complex is made by the stepwise addition of single sugar residues from sugar nucleotides, such as GDP-mannose, to the lipid in the membrane of the endoplasmic reticulum (ER). The completed oligosaccharide is then transferred as a unit from the lipid to the growing polypeptide chain. In the final stage of assembly, the peptide-bound oligosaccharide is modified, sometimes extensively, to yield the final glycoprotein.

Studies on the first stage of glycosylation suggest that sugar nucleotides are utilized at the cytoplasmic face of the ER. Hanover and Lennarz showed that the sugar nucleotide precursors of oligosaccharide-lipid are not able to penetrate the ER membrane^{1,2}, and in experiments using proteases to probe the organization of the enzymes of oligosaccharide-lipid synthesis, a number of enzymes have been shown to be sensitive to protease digestion from the cytoplasmic side of the membrane^{1,3,4}. These include enzymes that occur early and late in the pathway, as well as three which use sugar nucleotides directly. In addition, no enzymes were found that are sensitive to proteolysis only from luminal side of the membrane³. These experiments do not show, however, where the catalytic sites of the enzymes reside. The cytoplasmic protease-sensitive sites could represent the catalytic sites, but the data are also consistent with transmembrane enzymes having cytoplasmic protease-sensitive sites and luminal catalytic sites.

Although sugar nucleotides are utilized at the cytoplasmic face of the ER membrane, it is clear that sugar residues must cross the ER membrane during the assembly of oligosaccharide-lipid since the transfer of oligosaccharide from lipid to protein occurs within the lumen. How then do the sugar residues derived from cytoplasmic nucleotide sugars move across the ER membrane during the assembly of luminal oligosaccharide-lipid? One possibility is that glycosyltransferases carry the sugar residues from cytoplasmic sugar nucleotides across the membrane and add

them to growing oligosaccharide chains on the luminal face of the membrane. Such a mechanism is likely for the synthesis of the second intermediate in the pathway, dolichol-pyrophosphoryl-(N-acetylglucosamine)₂. This compound is only found facing the ER lumen^{1,5}, and is synthesized by an enzyme which is sensitive to proteolysis from the cytoplasmic side of the membrane^{1,3}. Because neither the nucleotide sugar precursor, nor the intermediate itself, can cross the membrane, glucosamine residues are probably transported across the membrane as an integral part of the glycosylation process.

A second possibility is that sugar residues are transported by the transmembrane movement of the lipid-linked intermediates themselves. This possibility has been ruled out for several oligosaccharide-lipid intermediates^{1,5}. The recent studies of Hasselbeck and Tanner⁶, however, suggest that dolichol-phosphate-mannose (Dol-P-Man), an intermediate which donates mannose residues to growing digosaccharide-lipids, may serve to transport mannose residues into the lumen. These workers have presented evidence for the transmembrane movement of Dol-P-Man in reconstituted phospholipid vesicles containing purified Dol-P-Man synthetase from yeast. This translocation is almost certainly protein mediated, as similar compounds do not flip spontaneously in model membranes⁷. If the same translocation occurs *in vivo*, then Dol-P-Man might be made on the cytoplasmic face of the ER membrane, and then flip so that it can serve as a mannose donor in the assembly of luminal oligosaccharide-lipid. Confirmation of this model will require the direct probing of the orientation of this compound in the ER membrane.

After transfer of the fourteen-sugar oligosaccharide from lipid to the polypeptide chain, the complex is transferred to the Golgi apparatus. There processing of the oligosaccharide takes

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place using a different mechanism for exporting sugar residues. In this case, both the glycosyltransferases and the glycoprotein substrates are found within the lumen⁸⁻¹⁰. The sugar nucleotides are transported across the membrane and are then used by the luminal glycosyltransferases, as shown by the specific uptake of nucleotide sugars into Golgi vesicles^{2,11}. Luminal utilization of these compounds in the Golgi is also suggested

by the fact that the nucleotide products of the glycosylation reactions are found within the lumen^{2,11,12}.

Future investigations should show whether topographical considerations can explain the involvement of lipid-linked oligosaccharide assembly. It will also be of interest to see whether similar transport mechanisms are used during the assembly and secretion of other types of complex carbohydrates. □

in *E. coli* is still basically unknown but a possible clue may be found in the observation of Linn (University of California, Berkeley) that in induced cells, a higher molecular weight variant of DNA polymerase I can be isolated that possesses lower fidelity of replication.

Another damage-inducible system is the adaptive response of *E. coli* to the effects of simple monofunctional alkylating agents. Pretreatment with low doses of these agents leads to a decrease in both the lethal and mutagenic effects of a subsequent challenge dose. Although both effects are controlled by the *ada* locus, adaptation for mutagenesis and for survival appear to occur by different mechanisms. The principal premutagenic lesion produced by methylating agents in DNA is *O*⁶-methylguanine. Lindahl's group (ICRF, London) have shown that the adaptive response to mutagenesis involves the induction of a 'suicidal' DNA methyltransferase which transfers the methyl group from *O*⁶-methylguanine residues to a cysteine residue in the enzyme, and thereby inactivates it.

*O*⁶-methylguanine residues which have not been repaired before passing through the replication fork will pair with thymine at a high frequency. As discussed by Schendel (University of Connecticut), this base pair may then become a substrate for the post-replicative mismatch repair system, as has been recently discussed by Karran and Marinus (*Nature* 296, 868; 1982). Thus the final mutagenic outcome of a premutagenic *O*⁶-methylguanine will depend on the relative rates of demethylation by the methyltransferase, DNA replication, mismatch repair and enzymatic methylation of daughter strands at GATC residues [which will prevent the mismatch repair system from being able to identify the daughter strand (Radman, Free University of Brussels)].

3-methyladenine is a potentially lethal lesion which in wild-type *E. coli* is rapidly removed by a constitutive enzyme 3-methyladenine-DNA-glycosylase I. During adaptation, however, a similar enzyme, 3-methyladenine-DNA-glycosylase II, is also induced. The role of this enzyme was not at first apparent, but one has now been put forward by Lindahl and Karran. 3-methylguanine is also a potentially lethal lesion, but it is formed at a much lower frequency than 3-methyladenine. After removal of 3-methyladenine (but not 3-methylguanine) by the constitutive enzyme, however, 3-methylguanine does constitute a major remaining lesion. The inducible glycosylase has a broad substrate specificity, acting on 3-methyladenine, 3-methylguanine and 7-methylguanine, and Karran and Lindahl thus suggest that its major role is to

Inducible responses to DNA damage

from Bryn Bridges and Alan Lehmann

THE idea that damage to DNA might call forth expression of specific cellular functions has flowered during the last ten years into an absorbing and productive area of research, particularly in the bacterium *Escherichia coli*. The current status of the field was the subject of a recent conference in Toulouse*. We now know that damaged DNA in a bacterial cell may induce many functions, ranging from prophage induction to DNA repair, error-prone DNA replication and the production of multinucleate filaments. The first workshop held on this subject in 1975, recalled by Witkin (Rutgers University), was notable for a preponderance of phenomenology and speculation. Now we understand some aspects, particularly control mechanisms, in very great detail.

The most important set of inducible functions in *E. coli* (often referred to as the SOS functions) is under the control of genes *recA* and *lexA*. The LexA protein acts as a repressor for almost all the genes known to express inducible functions (*din* genes), including both *recA* and the *lexA* gene itself (Little, University of Arizona; Brent, Harvard University). The sequences of the operator regions of many *din* genes are now known. Small differences in these sequences are probably responsible for their different sensitivities to induction.

The mechanisms of *recA-lexA* control have recently been explained in these columns (see S. Sedgwick & G. Yarranton *News & Views* 276, 606; 1982). It now seems likely that the signal that activates RecA protein into a specific protease capable of cleaving the λ repressor (Roberts, Cornell University) and LexA protein (Little) is single-stranded DNA (Weinstock, Frederick Cancer Research Center), unusually long lengths of which are likely to be present after DNA damage has been sustained. More light is now being thrown on the functions of some of the inducible gene products. RecA protein, for

example, can act not only as a protease but also as a single-stranded DNA-dependent ATPase, a DNA-unwinding or -annealing enzyme, and it can also catalyse the formation of D loops and strand exchanges in DNA (Lehman, Stanford University; Howard-Flanders, Yale University). Whereas the formation of D loops can occur at either end of a piece of single-stranded DNA and does not require perfect sequence homology (Radding, Yale University) branch migration occurs only in the 3' to 5' direction. Although it is generally sequence specific, RecA protein-catalysed branch migration can pass successfully over mismatches, small deletions and pyrimidine dimers (Radding; Lehman). In an intriguing brief presentation, Sief (Paris) pointed out some homologies between the sequence of the *recA* and the SV40 large T-antigen genes and between the functions of their products.

The 'UV endonuclease' of *E. coli* requires the products of genes *uvrA* and *-B* (both inducible) and *-C* in order to repair nick-irradiated DNA (Rupp, Yale University; Seeberg, Norwegian Defence Research Establishment). Rupp revealed that the complex of these gene products incises damaged DNA, cutting between the seventh and eighth bases on the 5' side of a pyrimidine dimer and between the third and fourth bases on the 3' side, thus raising the possibility that the enzyme complex can not only incise near damage but also excise the damaged region.

The inducible *umuC* gene of *E. coli*, which is absolutely required for UV mutagenesis, actually specifies two proteins, of molecular weights 45,000 and 16,000 (Walker, MIT). Walker and Kato (NIH) agreed to propose that the gene for the larger be referred to as *umuC* and that for the smaller (which is upstream) as *umuD*. Two apparently similar products are formed by the *muc* gene of pKM101, the mutator plasmid found in many of the Ames *Salmonella* mutagenicity tester strains. The mechanism of UV mutagenesis

* A conference on 'Inducible responses to DNA damage in prokaryotes and eukaryotes' was held on 5-8 May 1982, sponsored by the French Ministry of Research and Technology.

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increase survival by removing 3-methylguanine from alkylated DNA.

An intensive search for an analogous 'adaptation' system in mammalian cells is taking place. Several years ago Pegg identified an *O*⁶-methylguanine-demethylating enzyme in rat liver, and both he in collaboration with Montesano (IARC, Lyon) and Verly (University of Liege) have shown that this enzyme works in a similar way to the *E. coli* enzyme, transferring the methyl group to a cysteine residue of an acceptor protein, which is probably the enzyme itself.

The work of Montesano and collaborators and others over the past few years has shown that the ability to remove *O*⁶-methylguanine from rat liver can be induced by a pretreatment with alkylating agents, but the induction mechanism is probably different from that in *E. coli*. Inducers are not confined to those agents which produce *O*⁶-methylguanine in DNA (in contrast to *E. coli*), and it is possible that the increased activity of the methyl-transferase may be part of a generalized enzyme induction associated with the increased cell proliferation which results from treatment with inducing agents. In cultured cells, in some laboratories (Boyle, Patterson Laboratories, Manchester; Laval, Institut Gustav Roussy, Villejuif) but not others (Karran, MRC, Sussex), it has proved possible to reproduce the observations of Samson and Schwartz (*Nature* **287**, 861; 1980) that pretreatment with alkylating agents gives rise to an increase in survival on exposure to a subsequent challenge. Laval and co-workers have now also obtained evidence for a decrease in the mutagenic effect by pretreatment. It will be necessary to demonstrate that these cellular effects are really due to alterations in the appropriate enzyme activities in order to be convinced that the systems in mammalian cells are really the same as those in *E. coli*.

A fascinating but bewildering series of responses is being discovered after treatment of cultured cells with agents that damage DNA or block DNA replication. It has long been known that DNA damage in the host cell enhances the survival of infecting damaged viruses, a phenomenon analogous to Weigle reactivation of bacteriophage (one of the SOS functions). More recently it has been shown that this can also be accompanied by an increased mutation frequency in the infecting viruses. By sequencing some of the UV-induced mutants produced in SV40, Sarasin (Institut Gustav Roussy, Villejuif) has shown that they are usually single-base pair substitutions which occur opposite possible pyrimidine dimer sites. Oishi (University of Tokyo) described some elegant work showing that a protein produced in UV-irradiated cells could stimulate DMSO-induced differentiation of Friend cells. This was shown first by fusing UV-irradiated cells with DMSO-treated Friend cells and scoring for dif-

ferentiation in the hybrids. Subsequently extracts from UV-irradiated cells were able to produce the same effect in permeabilized DMSO-treated cells and this activity was partially purified. Other DNA damage-inducible phenomena include the induction of plasminogen activator in xeroderma pigmentosum cells (Ben-Ishai, Technion, Haifa), DNA ligase in human fibroblasts (Mezzina, Institut Gustav Roussy, Villejuif), gene amplification of the dihydrofolate reductase system in hamster cells (Tlsty, Stanford University) and of an Ia antigen in B lymphocytes (Herrlich, Kemforschungs Zentrum, Karlsruhe). However, an extensive search for an inducible error-prone repair system

producing increased cellular (as opposed to viral) mutagenesis with properties similar to that in *E. coli* yielded results that were entirely negative (Rossman, New York Medical Center).

Although the meeting was outstanding in terms of its scientific content, it is sad to report that the French Government is again insisting that its subjects present their papers in French. This, coupled with the customary inadequate simultaneous translation of specialized biological material into English, will have done much to minimize the impact of some really excellent work by French scientists on colleagues from countries where working knowledge of that beautiful language is lacking. □

Back-arc geodynamics

from Peter Barker

THE balance of force controlling extension behind subduction zones is still not understood, and for very good reasons: the complexity and small size typical of back-arc regions makes them resistant to analysis at a level of exploration more than adequate for the main ocean basins, and even those that are fully mapped show a perplexing range of histories and tectonic styles. Within this confusion survives a broad spread of dynamic models, from the simple one which maintains strict analogy to the generally accepted major plate model to those which claim, in essence, that the much smaller size of back-arc regions ensures the importance, dominance even, of forces quite undetectable in the behaviour of the major plates. A recent conference* most usefully and entertainingly arranged for a large proportion of the known data to confront the most recent dynamic models. As usual, the data won, but some models fared much better than others and the eventual comprehensive solution may not be too distant.

Additions to the data set included detailed aeromagnetic surveys north of New Zealand (Malahoff, NOAA), which have revealed two active RRR triple junctions in the North Fiji Basin and measured the current spreading parameters in the Havre Trough. A re-assessment of the hitherto confusing West Philippine Basin magnetic data by Lee and Hilde (Texas A & M University) convincingly identified an early (60–45 Myr) episode of fast NNE–SSW extension followed (45–35 Myr) by slow N–S spreading from the same centre. The back-arc nature of the early episode remains in doubt.

The recent notion that arc volcanism and back-arc extension alternate in time was seriously questioned by several contributors. A more restricted version, that arc volcanism ceases when back-arc rifting begins, was also queried, on the grounds that products of early rift-stage volcanism would be least accessible, through subsequent burial. Several arcs (Iwo-Jima Ridge, Mariana, South Sandwich) were shown to occupy just such an early-rift position, on the oldest crust formed during the most recent 'back-arc' spreading episode. Stern (University of Texas at Dallas) and Rubin (USGS) suggested that some arc volcanism associated with early back-arc rifting was alkaline, as in other rift environments.

Hilde and co-workers (Texas A & M and Tokyo Universities) noted a striking correlation between trench depth and the age of the subducting slab, and that where back-arc basin floor was being subducted the trenches were anomalously deep. Since subduction rate and slab age are also strongly correlated back to at least 150 Myr, it seems clear that oceanic lithosphere continues to develop with age, so that an age-dependent slab pull force should form part of any dynamic model. The anomalous depths of West Pacific back-arc basins, reflected in the trench depths, coincide in extent with a long-wavelength geoidal high (discussed also by Bostrom, Seattle) and both may result from the accumulation at depth of cold, dense ex-Pacific oceanic lithosphere by long-term subduction. Also on the theme of vertical isostatic response, Kobayashi (Tokyo University) explained a rapid initial subsidence of the Shikoku Basin as decoupling from a fore-arc abnormally elevated by a strong subducting slab, and

*Geodynamics of back-arc regions', the 4th annual symposium of the Geodynamics Research Program of Texas A & M University, College Station, held 29–30 April 1982, was convened by R.L. Carlson (Texas A & M) and K. Kobayashi (Tokyo) and co-sponsored by IUGL, WGB. The 40 papers delivered will be published in *Tectonophysics* in 1983.

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Le Pichon (University of Paris) suggested how the ultimate closure of a young, sedimented back-arc basin might follow naturally from its thermal and stress history.

Several speakers emphasized that no single tectonic model available accounted for all back-arc regions. Sleep (Stanford University) argued that the same forces governed back-arc evolution as controlled major plate motions, with much of the variety and relative instability of back-arc behaviour stemming from the different boundary conditions imposed by the surrounding major plates, the small sizes of back-arc platelets and the sharp changes in stress and strength across them. The meeting apparently agreed: little favour was found with the 'local' class of model (which explains back-arc extension by mantle diapirism or viscous drag, produced by the motion of the descending slab), because it fails to explain the distribution of extension in time and space, and because of the unreasonable mechanical properties required.

The difficulties of the other class of model, which stresses the significance of

'absolute' plate motion, were well described by Taylor (Hawaii Institute of Geophysics) and Carlson (Texas A & M University). In the Mean Hotspot Reference Frame, nearly all trenches are either stationary or migrating slowly oceanwards (described as 'roll-back' of the hinge of subduction), suggesting that the subducting slab becomes an anchor in the mantle. Back-arc extension occurs when the major plate behind the trench is retreating, rather than overriding. This simple and satisfying picture of back-arc extension as passive response explains most examples, but not the more complicated New Hebrides-Tonga and Scotia Sea regions, where back-arc extension and roll-back both seem excessively large. Nor is it clear why many characteristics of back-arc extension should be found in the Altiplano of Peru or the mid-Cenozoic Basin and Range Province where the continental plate is advancing rather than retreating. It was interesting, however, that the meeting appeared to consider these as anomalies to be explained away, rather than as reasons for outright rejection of the roll-back model. □

sources, on the maximal rate of turnover ($V_{\max}$) and on the concentration of actin required to achieve half this maximal rate (K_m). The use of myosin subfragments (soluble myosin heads) permitted the results to be analysed in terms of solution kinetics, a distinct advantage over an earlier paper¹³ where insoluble myosin filaments were used. Care was taken to ensure that tropomyosin remained bound at all times.

The most straightforward results were those obtained with vertebrate skeletal tropomyosin (40 mM KCl; 2 mM ATP; 3 mM $MgCl_2$). An approximately tenfold reduction in $V_{\max}$ (in comparison with results with pure actin) was accompanied by a tenfold decrease in K_m (that is, a tenfold increase in apparent affinity). Skeletal muscle tropomyosin thus acts as a non-competitive inhibitor, increasing the number of undissociated actin-myosin complexes; there is therefore no direct competition between myosin and tropomyosin for the same binding site, in agreement with the conclusions of Eaton⁹ but contrary to the steric blocking model. Sobieszek emphasizes that the changes in $V_{\max}$ and K_m do not imply changes in either the true binding constant of actin for myosin or in the effective number of myosin-binding sites on actin. Instead he envisions an increase in the half life of the ternary complex to account for the inhibition. This may well be true but it would have been nice if the ATPase studies had been conducted in parallel with direct binding studies in identical conditions, using the approach of Chalovich and Eisenberg¹².

The entire machinery of thick filament regulation resides on the myosin molecule. Scallop adductor muscle can be switched on by direct calcium binding to its regulatory myosin¹⁴. In variants of the steric blocking model proposed for such regulation, a subunit of myosin, the regulatory light-chain, physically blocks attachment to actin. Vibert and Craig (*J. molec. Biol.* **157**, 299; 1982) describe three-dimensional reconstructions of scallop thin filaments decorated with scallop myosin subfragments which may shed light on this mechanism. Two distinct forms of 'arrow-head' patterns can be produced, depending on whether or not the scallop myosin fragment retains its complement of light chains¹⁵ — 'barbed' (with light chain) or 'blunted' (without light chain). In Vibert and Craig's reconstructions, extra material can clearly be seen at the portion of each subfragment furthest away from actin in the 'barbed' filament. It is natural to assume that this material represents the regulatory light chain, absent from the subfragment used to obtain the 'blunt' images¹⁶, although one cannot rule out a partial contribution from the heavy chain (which may be of different lengths in the two types of subfragment). If this interpretation is correct, it is doubtful whether the regulatory light chain (albeit 10 nm long in solution¹⁷) could stretch from the distal

Retreats from the steric blocking of muscle contraction

from Peter Chantler

VARIANTS of a steric blocking model have been put forward as a basis for the control of both thin filament- and thick filament-regulated muscles. The model proposes that in response to calcium binding by troponin, tropomyosin moves from a blocking position on the edge of the actin long-pitch groove to a non-blocking position deeper in the groove. It arose primarily from structural studies on thin filament-controlled muscles — the X-ray diffraction patterns of Huxley, Haselgrove, Parry and Squire¹⁻³ coupled with electron microscopic and three-dimensional reconstruction techniques^{4,5}. Although the geometry of the model has altered by approximately 180° as a consequence of the more recent structural studies of Seymour and O'Brien⁶ and of Taylor and Amos⁷, the basic premise has remained unchanged. Initial biochemical studies in the presence of low levels of MgATP, such as those of Bremel and Weber⁸ concerning rigor complex formation, were, to some extent, consistent with such a model. More recently however, the biochemical evidence has revealed some difficulties. Saturation binding of myosin heads was found to increase the affinity of tropomyosin for actin in the rigor state^{9,10}, and many groups have shown that tropomyosin inhibits or elevates the actin-activated

ATPase of myosin subfragments depending on the source of tropomyosin. Most recently, it has been found that the binding constant of myosin heads for fully regulated actin (actin/tropomyosin/troponin = 7:1:1), in the presence of MgATP, is independent of the presence or absence of calcium^{11,12} — a property totally at odds with the steric blocking mechanism as originally conceived.

Two recent papers in *Journal of Molecular Biology* (**157**; 1982) present new challenges to the model. One (Sobieszek, Austrian Academy of Sciences) may be added to the above list of biochemical experiments that conflict with classic steric blocking. The other (Vibert and Craig, Brandeis University) is a structural paper that leaves the distinct impression that a type of steric blocking mechanism — in this case by regulatory light-chain subunits of myosin — may not be tenable as an explanation of thick filament regulation.

Sobieszek (*J. molec. Biol.* **157**, 275; 1982) has taken advantage of the hyperbolic relationship between the steady-state actin-activated ATPase and actin concentration in order to study the modifying effect of tropomyosins, from various

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portion of the myosin head to the actin-binding region thus making a steric blocking action of the regulatory light chain, during regulation, highly unlikely.

The reconstructions of the decorated thin filament clearly indicate a continuous axial ridge of density which the authors interpret as corresponding to tropomyosin; the density is absent from the reconstruction when tropomyosin-free actin is used instead of thin filaments. This interpretation yields a thin filament geometry that corresponds most nearly to the position of tropomyosin observed in recent studies of vertebrate skeletal thin filaments^{6,7}. The conservation of tropomyosin structure and location in a thick filament-regulated muscle is intriguing because tropomyosin is not required for, and does not influence, *in vitro* tests of regulation¹⁴; furthermore, a functional troponin complex has not been demonstrated in the scallop adductor muscle. Comparative reconstructions of decorated thin filaments have a serious weakness in that they are constrained to the rigor state where tropomyosin occupies a non-blocking position. It would be of interest to define the position of tropomyosin in relaxed scallop thin filaments and so explain the changing layer-line intensities observed in X-ray diffraction patterns of thick filament-regulated muscle — and interpreted as a stimulation-dependent movement of tropomyosin¹⁸.

Steric blocking, as originally envisioned, has reached its demise. However, we should not throw out the baby with the bath water. It is possible that during thin filament regulation, a subsequent rotation of the myosin head on the actin subunit is sterically blocked by tropomyosin. But

even if this is the case, things are still not that simple. To explain why actin/tropomyosin can stimulate higher rates than actin alone⁸, there must be a further step. This could take the form of conformational changes in actin induced by

tropomyosin or (much more exciting!) tropomyosin-induced changes in the myosin head. And alternatives to direct steric blocking mechanisms during thick filament regulation? Well, that's another story. □

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Why honeycomb weathering?

from Peter J. Smith

CHARLES DARWIN is sometimes credited with being the first to record its existence, although a close reading of the relevant text¹ hardly substantiates the claim. The point is barely worth pursuing, however, for the phenomenon is so widespread that it must have been observed many times before Darwin ever lived. It has certainly been observed many times since. Thus it can be seen in West German sandstone, Chilean agglomerate, New Zealand greywacke and Elba granite. Others have noticed it in Antarctic gabbro, Normandy schist, Hawaiian pahoehoe lava and Egyptian nodular limestone. Moreover, it is not entirely unknown in Corsican gneiss, Australian mudstone, American rhyolitic tuff and West Indian diorite.

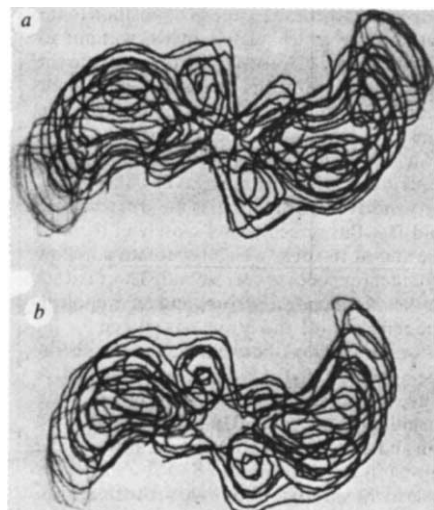
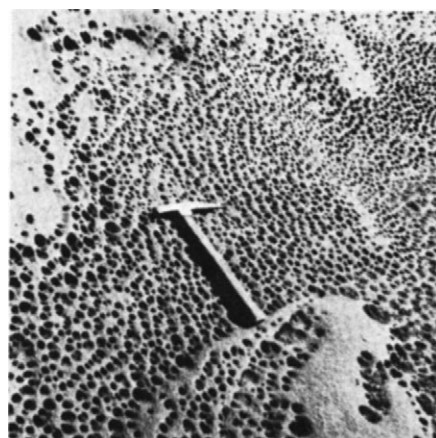
With so many independent observations of so many examples in so many different places, it is perhaps not surprising that it has come to have a variety of names. The French tend to call it alveolar weathering, although others have used the terms fretting, stone lattice and stone lace. With a remarkable lack of sensitivity, it has even been called miniature tafoni, which means to say miniature caverns. But the most common, and certainly the most aptly evocative, name is honeycomb weathering; and as the accompanying pictures show, the origin of that description needs no explanation.

The phenomenon itself does require explanation, however; and that is where the controversy starts. Late last century, Futterer² attributed honeycomb weathering in granitic rocks in Corsica to wind erosion, but Popoff and Kvelberg³ later took the view that it was a temperature effect. They envisaged the displacement of cold air in shady regions of the rock surface

by rising currents of warm air, and thus the generation of differential temperature variations. Cailloux⁴, on the other hand, claimed that the honeycombed surfaces in Corsica were remnants of Pleistocene frost damage.

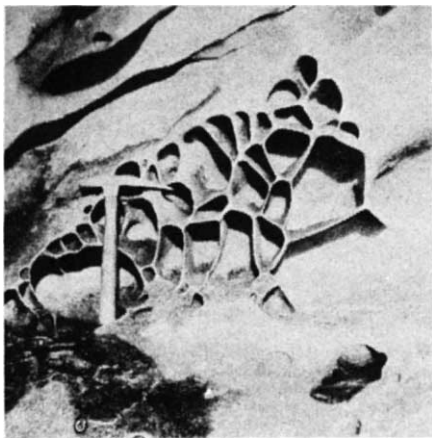
Other explanations depend on the idea of differential attack upon internal variations in structure or composition. Thus Palmer and Powers⁵, for example, claimed that honeycomb cavities in Hawaiian lava were produced by the erosive enlargement of vesicles. Bartrum⁶ attributed the cavities in New Zealand andesites to the dissolution of calcium carbonate cement surrounding volcanic clasts. But there are doubts about the general validity of this class of explanation, based on the fact that many, if not most, examples of honeycomb weathering are to be found in rocks that were apparently homogeneous to begin with. This difficulty led Blackwelder⁷ to suggest that the many

Honeycomb weathering on arkosic sandstone near Bellingham, Washington, USA. The cavities are 5–10 cm wide and generally have depths smaller than the widths. The overall pattern is fairly uniform because the sandstone surface was originally homogeneous.



Three dimensional reconstructions of scallop thin filaments decorated with scallop myosin subfragments: view along the helix axis. Two patterns can arise: *a*, barbed arrowhead (myosin light chain present); *b*, blunted arrowhead (myosin light chain absent). The region furthest from the central axis is more massive in the barbed structure, and Vibert and Craig propose that the extra material represents the light chain, (from *J. molec. Biol.* **157**, 1982)

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A particularly good example of the thin, delicate, weathering-resistant walls that develop between the cavities of honeycomb weathering.

honeycomb cavities in American desert rocks could be due to differential moistening of the rock surface, the hydration of feldspars at the moist sites, consequent exfoliation there, and the removal of the resulting debris by wind, rain and animal activity. On the other hand, Rondeau⁸ concluded in desperation that honeycomb weathering could not be accounted for by any process at work today. Ironically, this is the only 'explanation' that can be rejected with any degree of certainty, for honeycomb weathering can be seen on a sea wall built in France in 1898 and on sandstone blocks quarried in 1902 to build a railway embankment in Washington state.

For this background information we are indebted to Mustoe⁹, who also draws attention to Hume's¹⁰ suggestion that honeycomb cavities may result from the physical action of salt crystallization. To Mustoe, too, must go the credit for undertaking a proper study of the origin of honeycomb weathering in one particular rock formation, namely, the coastal Chuckanut Formation of Washington state. Thus whereas most previous writers on honeycomb weathering have been content simply to describe the phenomenon, a few have speculated on its cause and even fewer (if any at all) have tried to investigate its origin. Mustoe has now carried out the first detailed geochemical and mineralogical work on it.

He concludes that in the case of the arkosic sandstone of the Chuckanut Formation, Hume's explanation is basically the right one. The occurrence of honeycomb cavities only on coastal exposures, the observed distribution of salt spray and the salt crystals to which it gives rise, and, above all, the measured concentrations of soluble salts on the rock surfaces, leave little doubt that honeycomb weathering at Chuckanut results from the evaporation of salt water deposited by wave splash. Moreover, the erosive action of the salts is evidently physical rather than chemical, for the small amounts of eroded

sand left within the cavities are disaggregated grains with virtually no indication of chemical alteration or dissolution.

But even if salt is responsible, the problem is not entirely solved. The existence of honeycomb weathering depends not only on the formation of cavities but also on the failure of the weathering to proceed to the point at which the walls between the cavities are also eroded away, leaving no honeycomb pattern at all. Mustoe's findings on this are consistent with the view that chemical action plays no large part in the proceedings. Thus there is no mineralogical evidence of the hardening of the cavity walls by the infiltration of chemically derived cement, but there is evidence (both visual and chemical) of a covering of algae. What apparently prevents the cavity walls from disappearing is the development of a thin organic coating that presumably either acts as a physical barrier against salt spray or, by keeping the surface moist, retards the evaporation of saline solutions. Either way, it is pretty successful.

Of course, what applies to the Chuckanut Formation may or may not apply to the honeycomb weathering observed in the remarkably wide range of environments throughout the world. Indeed, on the face of it, it seems highly unlikely that a mechanism valid in a temperate coastal region would be appropriate to arid, tropical or Arctic conditions at an inland site. Yet the argument is not all one way, for even at inland sites there are possible sources of salt in migrating fluids or even in the rocks themselves. For the time being, however, questions about honeycomb weathering beyond Chuckanut must go unanswered.

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100 YEARS AGO

A RAPID-VIEW INSTRUMENT FOR MOMENTARY ATTITUDES

THE wonderful photographs by Muybridge of the horse in motion and those by Marey of the bird on the wing induced me to attempt the construction of apparatus by which the otherwise unassisted eye could verify their results and catch other transient phases of rapid gesture. Its execution has proved unexpectedly easy, and the result is that even the rudest of the instruments I have used is sufficient for the former purpose; it will even show the wheel of a bicycle at full speed as a well defined and apparently stationary object. This little apparatus may prove to be an important instrument of research in the hands of observers of beasts, birds and insects, and of physicists who investigate such subjects as the behaviour of fluids in motion.

The power of the eye to be impressed by a glimpse of very brief duration has not, I think, been duly recognized. Its sensitivity is vastly superior to that of a so-called "instantaneous" photographic plate when exposed in a camera, but it is of a different quality, because the impression induced at each instant of time

upon the eye lasts barely for the tenth of a second, whereas that upon a photographic plate is accumulative.

The instrument I commonly carry with me is a very rude one, but convenient for the pocket, and is shewn below. The duration of the exposure given by it under the action of its spring is the 360th part of a second, but the beginning and end of the exposure ought not to count, so little light passing through the edges of the pupil at those times that what is then seen is relatively faint and is disregarded. I estimate its practical duration at about one 500th of a second, and it is rather less when the finger acts with a sharp tap in opposition to the spring. The instrument is shewn without its sliding lid, which protects it from injury in the pocket. An arm turns through a small angle round C, its motion being limited by two pins. Its free end carries a vertical screen, which is a cylindrical (or better, a conical sheet described) round an axis passing through C perpendicular to the arm. As the arm travels to and fro, this screen passes closely in front of the end of the box, which is cut into a hollow cylinder (or cone) to correspond. There is a slit in the middle of the screen, and an eyehole in the centre of the end of the box. When the slit passes in front of the eyehole, and the instrument is held as in the Fig., a view is obtained. A stud, S, projects upwards from the arm, and an india-rubber band, B, passing round a fixed pin and a descending spoke of the arm acts as a spring in causing the stud S to rise through a hole in the side of the box, where the finger can press it like the stop of a *cornet à piston*. In using the instrument it is held in the hand as in the Fig., with the eyehole in front of the eye. Nothing is then visible, but on pressing or tapping the stud the slit passes rapidly in front of the eyehole, and the view is obtained. After this, the stud is released and the arm springs backwards, when a second view can be obtained, or the eye may be purposely closed for the moment.



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ARTICLES

Impact mechanics of the Cretaceous–Tertiary extinction bolide

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On Earth impact of ~10 km diameter, asteroidal or cometary objects the vaporized, melted and (<1 mm) solid ejecta transfer ~40–50% of their energy to the atmosphere, giving rise to a short possibly lethal (to large animals) heating pulse. Some 1–20 projectile masses of early, high-speed and highly shocked (diameter, <1 μ m) extraterrestrial-rich ejecta is lofted to altitudes of 10 km where it can be globally distributed. It is proposed that this material represents the global Cretaceous–Tertiary boundary layer. When in the upper atmosphere it may have caused decreases in solar tropospheric insolation and resulting major extinctions in biota.

A RECENT discovery by Alvarez¹ *et al.* and others^{2–8} has provided physical evidence for the impact of an extraterrestrial object that is contemporaneous with the Cretaceous–Tertiary (K–T) boundary (65 Myr). The evidence is contained in the 1–150-cm clay layer marking the K–T boundary. This clay layer is enriched relative to crustal abundances in noble metals such as iridium, osmium, gold, platinum, rhenium, ruthenium and palladium, and in addition in nickel and cobalt by factors of 5–1,000. It has a siderophile element abundance pattern that is compatible with a concentration of extraterrestrial material in the range of 1–21% (ref. 5). The layer, which marks this event, appears to be global in extent; it has been found in marine sediments in Gubbio, Italy; Stevns Klint and Jutland, Denmark; Caravaca, San Sebastian, and Zumaya, Spain; Biarritz, France; El Kef, Tunisia; Woodside Creek, New Zealand; Falls County, Texas⁷, and Deep Sea Drilling Project (DSDP) Hole 465A, Central Pacific and in nonmarine sediments in the coal bearing strata in Raton Basin, New Mexico. In addition, Smit and Klaver⁹ found millimetre-sized chondrule-like spherules of nearly pure potassium feldspar which may have cooled rapidly from the melt, which also suggests a possible impact origin of the layer.

The observed surface noble metal concentrations have been used to estimate the size of the impacting object or bolide¹. Using only the observed iridium abundances, which vary from 15 ng cm⁻² (Gubbio, Italy), 25 ng cm⁻² (Raton, NM) to 520 ng cm⁻² (DSDP465A), and the concentration of iridium in chondrites (0.5×10^{-6}), total minimum bolide masses of 0.2 to 0.6×10^{18} g or diameters from 5 to 16 km (density = 2.2 g cm⁻³) have been calculated. The kinetic energy associated with such objects $\sim 10^{30}$ erg ($\sim 10^{14}$ tons of TNT), comparable with that associated with the basin forming impacts on planetary surfaces¹⁰.

It is highly probable that objects of the above size range have impacted the Earth. Telescopic observations of the population of Earth-crossing asteroids and the post-mare record on both the Moon and the Earth suggest that the Earth could encounter a 10 km-size object of the order of every 10^8 yr (ref. 11). Moreover, collisions with objects of diameters of 10^3 km are inferred to have occurred on all the major planets since their formation because of the deviations of the angle of the spin axes of the terrestrial planets with respect to the ecliptic¹².

The major physical evidence which is lacking is the location of the impact crater site. The diameter of the crater would be in the range of 100–150 km. Craters of this size are observed on the Earth but they are not contemporaneous with the K–T boundary. If the impact occurred in the ocean, the crater would be more difficult or impossible to locate since one-half of the ocean floor has been subducted in the past 65 Myr.

Because the clay layer is at the K–T boundary, it has been argued that the impact event is a source of K–T extinctions of biota¹. The most compelling evidence for the correlation of the impact event with extinctions is contained in the marine sediment record of the phytoplankton^{1–4}. The correlation with the extinction of other biota is more controversial. According to the compilation of Emiliani *et al.*¹³, the extinctions of species in the classes of late Cretaceous genera were selective and limited to floating marine phytoplankton and zooplankton, swimming molluscs (ammonites and belemnites), swimming dinosaurs (ichthyosauria and pleiosauria), corals (shallow water), clams and flying and walking dinosaurs which include all land animals which had masses >25 kg.

Alvarez *et al.*¹ and others^{2,3,5} have proposed that physical and possible chemical⁴ effects of the impact of the bolide would directly induce massive extinction of species. These extinction mechanisms include: short-term heating of the atmosphere from impact energy¹³, shielding of the Sun by lofted fine impact ejecta¹ and a subsequent sharp cooling of the oceans and atmospheres¹⁴, a decline in photosynthesis rates¹, shock production of NO (refs 15, 16) lofting of several times the normal stratospheric complement of water into the stratosphere giving rise to either a terrestrial greenhouse¹³, or possible enhanced transmission of UV light due to decline in stratospheric O₃ levels¹⁶.

We have examined the mechanics of asteroidal, cometary and meteoroid swarm impact on the Earth to consider whether or not the impact process is compatible with the physical evidence and if so, what extinction mechanisms are implied. Specifically, we have considered the questions: (1) Is the extreme enrichment of projectile material (up to 20%) in the K–T boundary clay relative to ordinary impact breccias and melts on the Earth and the Moon consistent with the impact process? (2) What is the size range of possible impactors, if the mass of the material in the K–T boundary clay represents impact ejecta lofted to heights >10 km? (3) How is the kinetic energy of a large Earth impactor partitioned? How much energy is converted to heat in the solid Earth, how much is available to heat the ocean and atmosphere? (4) What is the temporal sequence of impact phenomena that could give rise to the diverse set of extinctions that have been observed?

Approach and scope

We have modelled the flows resulting from assuming normal impact of spherical projectiles onto layers of air, water and silicates. Eulerian finite difference numerical algorithms were used to solve the conservation equations of mass, momentum and energy, along with the appropriate equations of state^{17–21}.

The Earth and asteroidal bolides were assumed to be gabbroic

anorthosite, and cometary bolides were assumed to be water ice¹⁷. The Tillotson equation of state¹⁹ was used for these materials and is given by

$$P = \left[a + \frac{b}{(E/E_0\eta^2) + 1} \right] E\rho + A\mu + B\mu^2$$

Where P is the pressure, E the internal energy, ρ and ρ_0 are the density and initial density, and $\eta = \rho/\rho_0$, $\mu = \eta - 1$ and $a = 0.5$. The constant, b , is defined such that $(a + b)$ is the STP Gruneisen parameter, $\gamma = (\partial P/\partial E)_\rho/\rho$ and A is the bulk modulus. The constants b , B and E_0 are obtained by fitting Hugoniot data up to ~ 1 Mbar and Thomas-Fermi calculations in the 10^2 Mbar region²¹. The large volume change phase transitions which occur at ~ 0.15 Mbar in silicates are accounted for in the high-pressure form by the equation of state. The energy of transition is $0.013 \text{ Mbar cm}^{-3} \text{ g}^{-1}$. At high temperatures and low pressures, the equation of state is given by a form which approaches that of an ideal gas^{18,19}.

The computational algorithm accounts for finite strength of the materials. However, because the effective strength of bolides of the order of 10 km and the Earth on this size scale is low^{22,23}, the strength was assumed to be zero.

The ejecta trajectories and the amounts of mass and the energy contained in that mass on being launched to a given velocity were computed from the flow for each computational zone that had an upward directed velocity component at various times. The amount of high velocity (V) ejecta ($V > 10^4 \text{ cm s}^{-1}$) became invariant before the projectile was stopped by the target whereas the amount of material launched to lower speeds is still increasing at the end of our (early time) calculations. The high velocity ejecta is of greatest interest.

Calculations were carried out for impact velocities of 15, 25, 45 and 72 km s^{-1} . The range of assumed impactor densities were 2.93 (stony asteroid), 1.0 (comet nucleus), 0.1 and 0.01 (meteoroid swarm) and the Earth's density was assumed to be 2.93 g cm^{-3} . We specifically consider the penetration of a large bolide with the atmosphere, the ocean, the excavation of a transient cavity and the interaction of the resulting ejecta with the atmosphere.

Penetration of the atmosphere and ocean

The atmosphere and oceans do not significantly impede bolides having diameters of the order of 10 km, which is comparable with the scale height of the atmosphere (7.1 km) and the mean ocean depth (3.6 km). We have calculated the flow resulting from the interaction of asteroids, comets and distended swarms of objects into the atmospheres and oceans. Penetration depths can be conveniently defined as the distance (as measured in projectile diameters) which is required to deliver $\sim 99\%$ of the bolide energy into the atmosphere or ocean²⁴.

For example, a 1 g cm^{-3} body impacting at 72 km s^{-1} will be completely stopped in the atmosphere if its diameter is $< 0.17 \text{ km}$ whereas the maximum diameter of a swarm of objects with an effective density of 0.01 g cm^{-3} impacting at 72 km s^{-1}

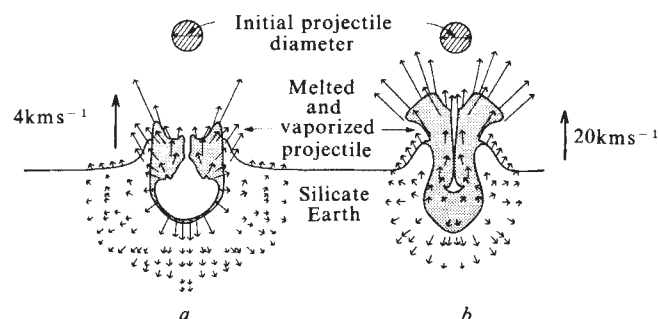


Fig. 1 Particle velocity flow field from a silicate projectile impacting a strengthless silicate planetary surface at 15 and 45 km s^{-1} . Flow fields at a , $\tau = 8.2$; b , $\tau = 3.1$ where τ is normalized time (see Fig. 3).

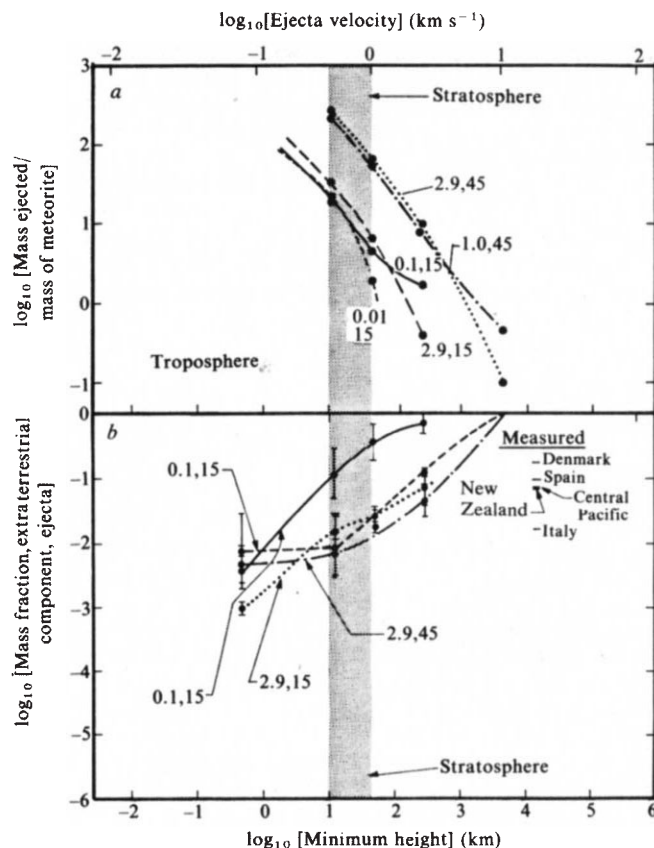


Fig. 2 $\log_{10}$ (mass ejected/mass of meteorite) (a) and $\log_{10}$ (mass fraction extraterrestrial component, ejecta) (b), plotted against $\log_{10}$ (minimum ejection height) (km) or $\log_{10}$ (ejecta velocity), (km s^{-1}). Typical mass fractions of extraterrestrial from various localities are shown. Ballistic ejection and no atmosphere is assumed.

would be 1.5 km (ref. 24). In the case of oceanic impact (25 km s^{-1}) 15 bolide diameters are required to stop an asteroid²⁴. For a given diameter object, we have shown that the penetration depths for either the ocean or the atmosphere is nearly independent of impact velocity. Not surprisingly then, a 10-km size object will surrender a negligible fraction of its kinetic energy on penetrating the atmosphere or ocean.

The ratio of the atmospheric penetration time τ_p characteristic ablation time τ_a is given by

$$\frac{\tau_p}{\tau_a} = \frac{3}{4} \frac{\rho_a l_a C_R V^2}{\rho_m d Q}$$

where ρ_m ($\sim 2.5 \text{ g cm}^{-3}$), ρ_a ($\sim 0.001 \text{ g cm}^{-3}$), l_a (7 km) and d (10 km) are the bolide and atmospheric densities, atmospheric scale height and bolide diameter. Here C_R (~ 0.01) is the radiative ablation transfer coefficient and Q ($\sim 4 \times 10^{10} \text{ erg g}^{-1}$) is the energy of ablation²⁵. For an impact velocity of $V = 30 \text{ km s}^{-1}$, $\tau_p/\tau_a \sim 0.005$. The ablation time τ_a is generally 10^2 – 10^3 times longer than the penetration time, implying insignificant mass loss and energy transfer due to penetration of the atmosphere.

Impact cratering on the Earth

The primary transfer of momentum and energy to the Earth occurs on impact. Cratering flow fields induced by silicate bolides (2.93 g cm^{-3}) (Fig. 1) demonstrate that during initial penetration, meteoritical material lines the wall of the growing cavity. The pressures produced during this time are sufficient to melt ~ 10 ($V = 15 \text{ km s}^{-1}$) to ~ 90 ($V = 45 \text{ km s}^{-1}$) times the bolide mass and to vaporize 4 ($V = 30 \text{ km s}^{-1}$) to 15 ($V = 45 \text{ km s}^{-1}$) times the bolide mass. The effect of total bolide vaporization on the cratering flow is demonstrated in Fig. 1b. The maximum mass ejected from the crater with at least an

upward velocity such that it could be lofted to various altitudes in the absence of the atmosphere are shown in Fig. 2a for different impact velocities and densities. We conclude that in the absence of an atmosphere, 20 to several hundred times the bolide mass could be ejected to a height of at least 10 km depending on the impact scenario.

The initial ejecta has extraordinarily high extraterrestrial component (ETC) concentrations and high velocities. The concentrations of ETC versus time (Fig. 3) demonstrate that a peak in ETC occurs at $\tau \approx 2$ or when a solid bolide has embedded itself in the planetary surface about two projectile diameters. In the case of a swarm impact, the highest ETC concentrations occur on initial interaction ($\tau \leq 1.0$) and then decrease with time. Figure 3 demonstrates that the initial high-speed ejecta has an ETC ratio compatible with the K-T boundary material.

The mass fraction of ETC ejected from the crater with at least a given ejection velocity and the associated minimum ejection height in the absence of an atmosphere, is demonstrated in Fig. 2b to be similar to those measured at various sites where the K-T boundary layer has been sampled. Kyte *et al.*⁵ pointed out that the K-T boundary clay layer is remarkable in that it appears to contain as much as 20% ETC, far more than the usual $\sim 1\%$ meteoritic component found in the vicinity of terrestrial meteorite craters or in the impact breccias collected from the lunar surface. We conclude that the origin of the ETC in the K-T boundary layer is uniquely the high-speed early-time ejecta which is lofted to the stratosphere, and higher, and is distributed zonally in $\sim 10^1$ days and meridionally on a time scale of $\sim 10^2$ days²⁶. Most of the impact ejecta around both terrestrial and lunar craters is indigenous material resulting from enormously larger quantities of low-speed ejecta.

The energy partitioning which occurs on impact transfers $\sim 85\%$ of the initial kinetic energy into internal energy, which is largely carried in planetary surface ejecta, for solid impactors. In the case of effective low density ($0.1\text{--}0.01\text{ g cm}^{-3}$) swarm impactors, considerably less of the initial energy ($5\text{--}40\%$) is partitioned into planetary internal energy. For a representative, 30 km s^{-1} impact of an asteroid with the Earth $\sim 50\%$ of the energy resides in the ejecta having velocities $> 10^4\text{ cm s}^{-1}$ (Fig. 4). This value rises to 80% of the energy for swarm impact (effective densities 0.1 g cm^{-3}) at 30 km s^{-1} . The energy partitioning between internal and kinetic energy in the ejecta also shown in Fig. 4, demonstrates that most of the energy in the high-speed ejecta ($V > 10^4\text{ cm s}^{-1}$) is internal for asteroidal impacts.

We conclude that the impact of large objects on the Earth can produce flow fields that eject up to several hundred times the object mass into the atmosphere with velocities $> 10^4\text{ cm s}^{-1}$ and that this ejecta contains $\sim 50\%$ of the impact energy.

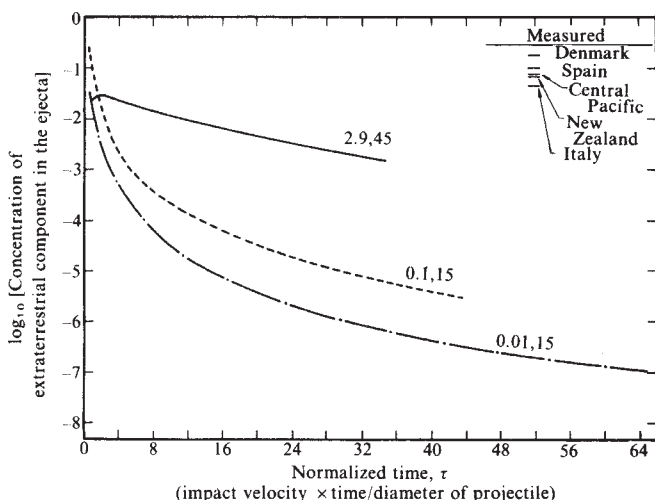


Fig. 3 $\log_{10}$ (mass fraction extraterrestrial component of ejecta), plotted against normalized time. Two values for each curve, for example 2.9, 45, indicate density of impactor and velocity.

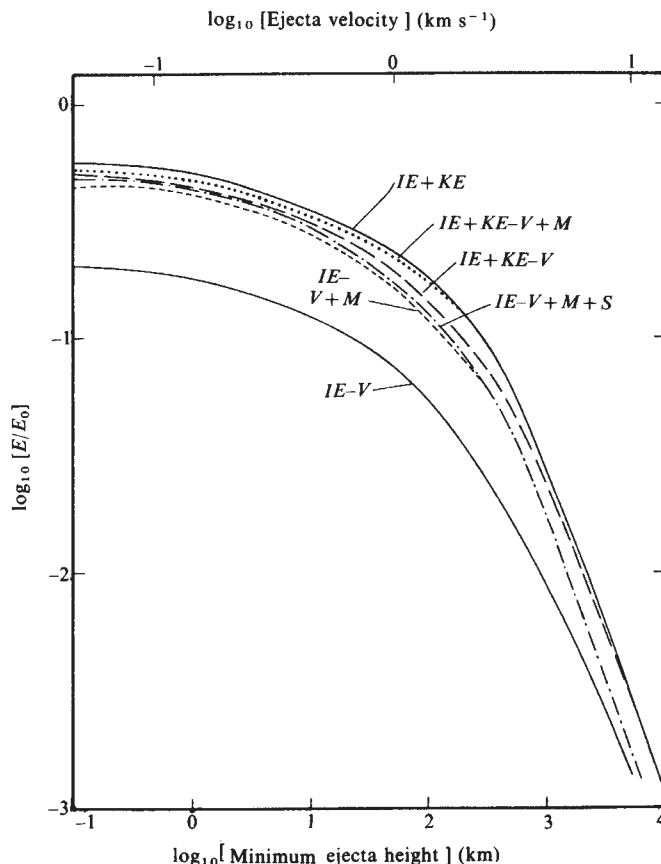


Fig. 4 $\log_{10}$ (energy of ejecta/initial projectile energy), plotted against $\log_{10}$ (minimum ejecta height), (km), or, $\log_{10}$ (ejecta velocity) (km s^{-1}), for silicate projectile impacting silicate Earth at 30 km s^{-1} . Ballistic throwout is assumed. $IE - V + M + S$ indicates internal energy in vapour, melt and solid ejecta, whereas $KE - V + M + S$ indicates kinetic energy of vapour, melt and solid ejecta, and so on.

However, this is not sufficient to establish whether the mass in the high-speed ejecta can be distributed globally and whether its energy can be transferred to the atmosphere. Both of these issues depend critically on the particle size distribution of the ejecta. This aspect of the problem has been considered by O'Keefe and Ahrens²⁴ and the results are summarized here.

To distribute ejecta globally, the residence time in the upper atmosphere has to be greater than several months. This implies particle sizes in the range of $1\text{ }\mu\text{m}$ or less. We believe the condensation products of vaporized bolide and terrestrial material is a source of such small particles. We have calculated²⁴ that depending on velocity and impactor density some $< 1\text{--}15$ times the mass of a bolide becomes impact vaporized material which is launched early in the flow at high speeds. Melted and highly shocked solid ejecta is also a source of small particles. The fraction of the melted particles in the $< 1\text{ }\mu\text{m}$ size range is difficult to estimate. However, for differences in droplet and air velocity of 10^4 cm s^{-1} , the maximum stable droplet diameter is $\sim 100\text{ }\mu\text{m}$. We estimate that the mass of melt particles contained in the $< 1\text{ }\mu\text{m}$ ejecta fraction is somewhat less than 1 bolide mass. In the case of solid ejecta, $\sim 10^{-3}$ of the mass ejected with velocities $> 10^4\text{ cm s}^{-1}$ would have a mean diameter in the range of $1\text{ }\mu\text{m}$ or less²⁷. Thus, the amount of solid ejecta having a diameter of $< 1\text{ }\mu\text{m}$ would also be < 1 bolide mass. From these arguments we suggest that $1\text{--}20$ times the bolide mass of particles $< 1\text{ }\mu\text{m}$ can be produced by impact.

The transfer of both the kinetic and internal energy in the ejecta depends on the particle size and velocity distributions²⁷. The kinetic energy of particles $\leq 10^0\text{ cm}$ in diameter is nearly totally transferred into thermal energy in the atmosphere by work done by drag forces, whereas particles with diameters $\geq 10^2\text{ cm}$ are inferred to be launched at speeds of $\leq 10^3\text{ cm s}^{-1}$,

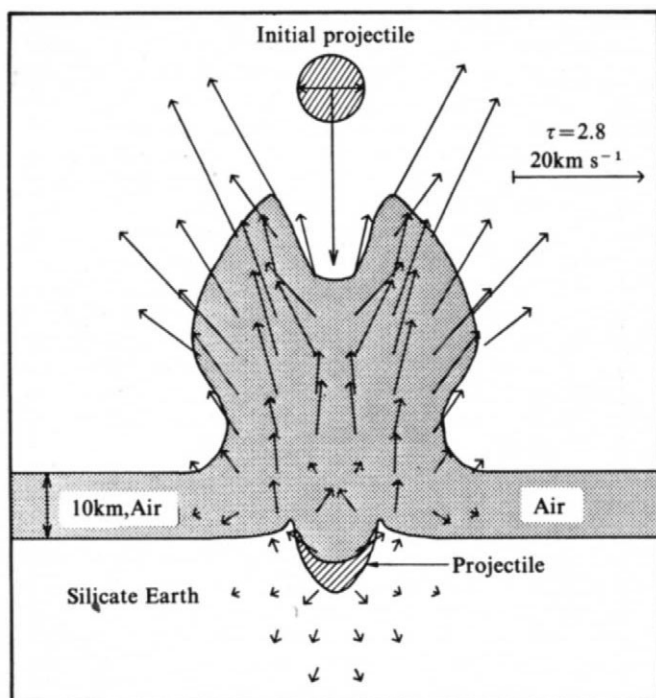


Fig. 5 Particle velocity flow field from a silicate projectile impacting a uniform 0.001 g cm^{-3} atmosphere overlying a silicate Earth at 5 km s^{-1} at a $\tau = 2.8$ when projectile has delivered $\sim 90\%$ of its energy to the target(s).

and subsequently these display essentially vacuum-like trajectories. These larger ejecta thus transfer little of their kinetic energy to the atmosphere. The internal energy of particles whose characteristic cooling time is greater than the residence time cannot be transferred to the atmosphere; this transfer can only occur for particles $< 0.1 \text{ cm}$ in diameter. From these arguments, estimates can be made of the coupling of the ejecta energy to the atmosphere.

For example, consider a 30 km s^{-1} impact of an asteroid. The fraction of internal and kinetic energy contained in the vapour, melt and solid ejecta having ejection velocities in the range $0.1\text{--}0.5 \times 10^5 \text{ cm s}^{-1}$ is given in Table 1. The kinetic energy of the solid ejecta launched at speeds between 0.1 and $0.5 \times 10^5 \text{ cm s}^{-1}$ contains some 5% of the projectile energy. Because this material will have particle diameters $< 10^1 \text{ cm}$ nearly 100% of the kinetic energy will be transferred to the atmosphere by aerodynamic drag. In contrast, the internal energy contained in the solid ejecta would not be efficiently transferred to the atmosphere because the relative amount of mass in particles $< 0.1 \text{ cm}$ diameter is small ($< 10\%$). Since in general the expected particle size of ejecta, initially melted or vaporized is expected to be comparable with, or smaller than, the solid phase ejecta, the kinetic energy contained in the ejected melt and condensed vapour would be transferred efficiently to the atmosphere. This kinetic energy in the $0.1\text{--}0.5 \times 10^5 \text{ cm s}^{-1}$ ejecta, represents some 9–11% of the kinetic energy of the bolide, is lost to the atmosphere as indicated in Table 1. The internal energy contained in condensed vapour ejecta and melt would also be effectively transferred to the atmosphere because the mean stable molten droplet size for velocity fluctuations of the order of 10^4 cm s^{-1} is $< 0.1 \text{ cm}$. We estimate a significant fraction of the impact energy (possibly as much as 40–50%) is transferred to the atmosphere by high-speed ejecta.

Implications of the ejecta–air interaction

The atmosphere has little effect on a large bolide during its initial penetration or during the subsequent induced motions of the cratering flow. However, as discussed above, the atmosphere strongly interacts with fine ejecta particles. To examine these effects further, we computed the cratering flow resulting from the impact of a 10-km diameter bolide onto a uniform

(10-km air layer) (density 0.001 g cm^{-3}) overlaying a solid Earth (Fig. 5).

On penetration of a 10-km diameter bolide through a uniform air layer a hole equal to the bolide diameter is immediately created. Although the interaction of the atmospheric bow shock with the Earth results in a series of wave reflections and Mach shocks which give rise to very high gas pressures ($\sim 0.1 \text{ bar}$) and internal energies ($\sim 10^{11} \text{ erg g}^{-1}$), the usual (Fig. 1) cratering flow fields (with ejecta emanating from the lip of the crater) result. During the penetration of the bolide into the Earth, the atmospheric 'hole' closes over the projectile. This occurs rapidly because the highly shocked air on the periphery of the 'hole' expands rapidly into the bolide path and gives rise to a rapid upward blow-off of the atmosphere^{28,29} (Fig. 5). The initial high-speed heavily shocked ejecta ($< 1.0 \text{ cm}$) emanating from the lip of the growing crater is entrained in the upward moving atmosphere at a velocity of ($\sim 10^0\text{--}10^1 \text{ km s}^{-1}$) and is lofted to altitudes of the order of 10 km. The very fine particles ($< 1 \mu\text{m}$) would have long residence times and can be distributed globally.

The millimetre to centimetre sized, initially molten objects, in this upward directed flow, are projected and lofted backwards along the initial bolide trajectory, cooled by radiation and conduction and then re-enter the Earth's atmosphere. These may be the tektites and microtektites. We suggest that the east–west orientation of their strewn fields may result from the preferential influx of bolides onto the Earth from the plane of the ecliptic.

Discussion and conclusion

During the initial penetration of the atmosphere and/or ocean relatively little mass or energy could be transferred from the K-T bolide. Although a water or silicate would have low strength largely as a result of either the intrinsic properties of icy partially devolatilized cometary material or because of previous impact history of the asteroids³⁰, neither tidal disruption nor aerodynamic forces²³ are likely to reduce the effective density (as proposed, for example, by Kyte) of such objects because they so rapidly encounter the Earth and readily penetrate the atmosphere. Although we have considered the impact of low density objects and equivalently, swarms of meteoroids, we recognize that no telescopic evidence for such highly distended objects are now available. Over the range of the velocities ($5\text{--}72 \text{ km s}^{-1}$) and impact densities ($0.01\text{--}2.93 \text{ g cm}^{-3}$) considered, the dominant mass and energy transfer occurs on impact with the Earth. We note that the shock pressures and accompanying internal energy densities achieved in cometary bolides would on impact decompose compounds such as cyanogens, which if brought to the Earth in significant quantities in comets might be considered⁴ a possible agent which could poison biota and give rise to extinctions. The flow fields induced by the impact of comets, asteroids or distended impactor impacts on the Earth can inject $\sim 10\text{--}10^2$ times the bolide mass in the atmosphere. In the case of ocean impact, water

Table 1 Fraction of total energy that ejecta transfers to atmosphere for a 30 km s^{-1} asteroidal impact

	Fraction of total energy in the $0.1\text{--}0.5 \times 10^5 \text{ cm s}^{-1}$ ejecta	Efficiency of transferring energy to atmosphere	Fraction transferred to atmosphere
Vapour—Internal energy	0.12–0.20	100	0.12–0.20
Melt—Internal energy	0.15–0.23	100	0.15–0.23
Solid—Internal energy	0.07–0.03	0.1	0.01–0.00
Vapour—Kinetic energy	0.01–0.01	100	0.01–0.01
Melt—Kinetic energy	0.05–0.03	100	0.05–0.03
Solid—Kinetic energy	0.05–0.05	100	0.05–0.05
Totals			0.39–0.51

masses of $\sim 10^{1-10^2}$ times the bolide mass are also injected into the atmosphere. The initial, high-speed ejecta which is lofted by the cratering flow is enriched in ETC material and has concentrations in the range of those measured in K-T boundary layer materials. We infer that this enriched material was highly shocked and consists of condensed vapour and melt and fine particles. This material would be initially entrained in the strongly heated atmosphere. Penetration of the atmosphere by the bolide, creates a hole in the atmosphere that is subsequently filled by a flow field that is inward and upward. The upward component of the flow field lofts the vapour, fine melted and solid ejecta to heights > 10 km. The millimetre to centimetre-sized melt droplets which are lofted by this mechanism re-enter the atmosphere and may represent microtektites and tektites. In contrast, the particles $\leq 1 \mu\text{m}$ which comprise $\sim 1-20$ times the bolide mass can be lofted to heights > 10 km and be distributed globally in several months. Calculations of the solar transmission reduction due to such dust injection³¹ show that 10^{16} g uniformly distributed over the Earth would reduce photosynthesis by a factor of 10^5 . Notably, this mass is only 10^{-2} times the mass of the estimated K-T bolide from iridium concentrations¹. An amount of dust injected of this magnitude would probably result in strong cooling of the troposphere and heating of the stratosphere²⁶.

In the case of a cometary impact on land or an ocean impact, a significant fraction of the ejecta lofted to high altitudes would be water. Undoubtedly much of this water would be rapidly condensed and rain-out. However, both strong decreases in the concentration of ozone and possibly triggering an enhanced terrestrial greenhouse may ensue. Large amounts of water

injected into the atmosphere might also reduce stratospheric residence times for fine solid particles by entraining them in the rain-out of the water. The passage of the bolide¹⁵ and the ejecta²⁴ through the atmosphere would produce high temperature shock waves, thus, NO. This might interact with the ozone layer and deplete it for times of the order of a decade. The atmosphere would be heated by both the shock waves produced by the bolide and ejecta and the transfer of internal energy from the fine ejecta. The fraction of bolide energy transferred to the atmosphere through this mechanism could be as great as 40–50%. This could, if not immediately reradiated to space, result in a global temperature increase of 30 K for a 10^{31} erg impact. Although the characteristic time scale for the radiative decay of such a temperature excursion might be only a matter of days, Emiliani *et al.*¹³ has argued that such a heat pulse would be intolerable to many biota (especially larger reptiles > 25 kg).

The impact of the K-T bolide gives rise to several phenomena and processes that can result in massive extinction of biota. Several mechanisms are probably required to explain the observed massive, but selective, extinctions both of marine and terrestrial animals at the K-T boundary.

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Dynamic observation of defect annealing in CdTe at lattice resolution

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Atomic-level events in the semiconductor material CdTe have been documented directly with real-time video recording, using a high resolution transmission electron microscope equipped with an image pick-up and display system. Among the various solid-state reactions observed were dislocation motion by slip, dislocation climb by diffusion and the annealing of several types of defects.

THE possibility of directly observing, and documenting, the motion of atoms in solids has been achieved in a few instances by taking sequential micrographs. The most notable of such

studies concerns atomic migration on surfaces, recorded on metals by field-ion microscopy¹ and on amorphous carbon substrates by scanning transmission electron microscopy². In only one case, to our knowledge, have television-rate, continuous recordings been made at the atomic level: namely, high-resolution transmission electron microscopy (HRTEM) observations of dislocation motion and twinning in gold³, though these corresponded to atomic columns rather than single

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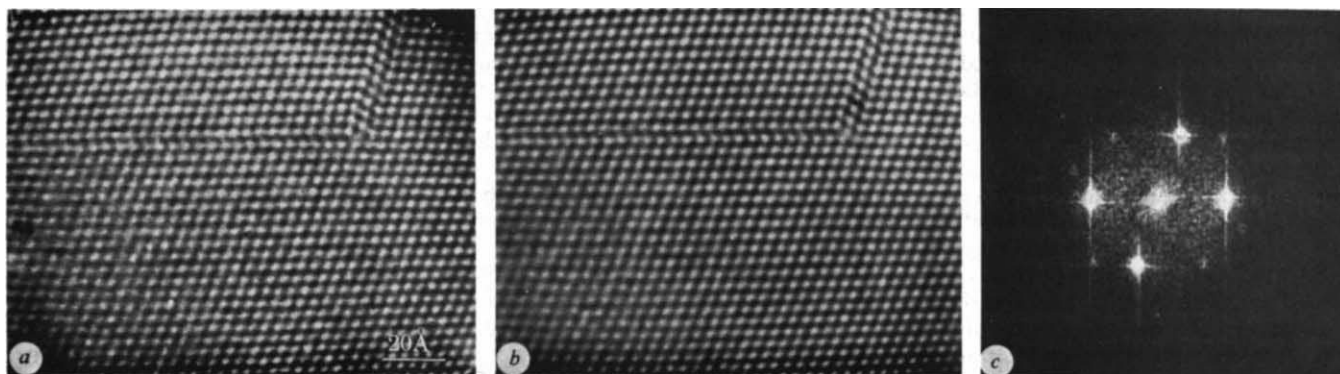


Fig. 1 *a*, Single frame of the television signal showing the intersection of an intrinsic stacking fault (horizontal) and an extrinsic stacking fault (inclined)—both faults are on {111} type planes. Each white spot represents the projection of a CdTe pair of atomic columns. Crystal thickness is estimated to be in the range 50–100 Å. *b*, The average of 10 frames, using the frame store and correcting for non-uniform television camera response. *c*, The diffraction pattern (Fourier transform) of the corrected image.

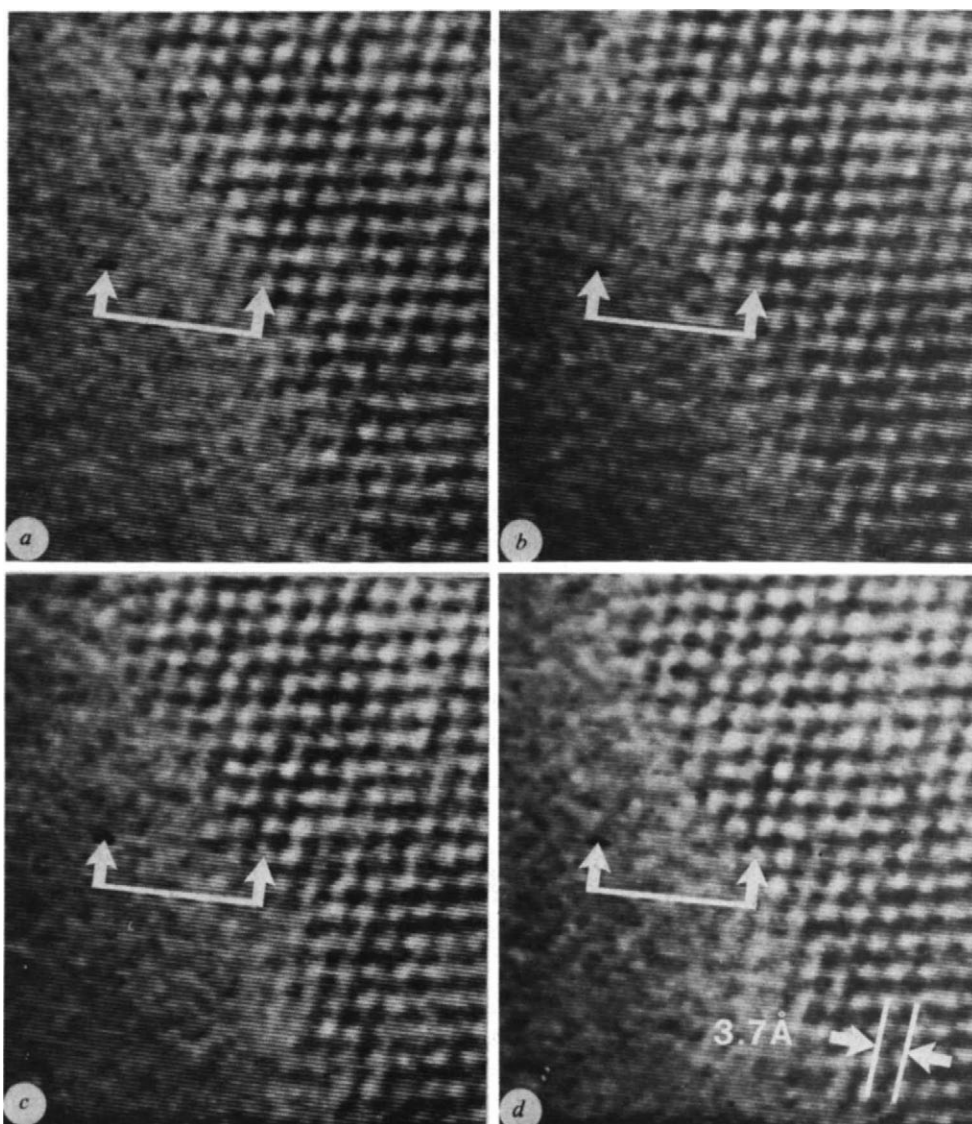


Fig. 2 Individual frames from a sequence where image spot changes are noticeable at the edge of the specimen. The same image spot is arrowed in each picture. Additional image spots appear in (*b*), (*c*) which are not present in (*a*). They also disappear again in (*d*). The time intervals are *a*, 0.00 s; *b*, 0.26 s; *c*, 0.40 s; *d*, 0.46 s.

atoms. The visual observation of 'atomic motion' (of projected columns of atomic pairs) at the edge of a CdTe specimen has been reported recently during investigations with a 120-kV HRTEM although video recording was not possible⁴. Publication of this work coincided with tests of the image pick-up and video recording system^{5,6} attached to the Cambridge University 600-kV HRTEM^{7,8}. It was then suggested that a CdTe specimen

should be observed in the Cambridge microscope using dynamic recording conditions: we now describe the results.

Imaging conditions

At present, the Cambridge microscope is limited to ~ 2 Å point resolution at 500 kV, although, at the magnifications used here, the effective resolution of detail recorded by the pick-up

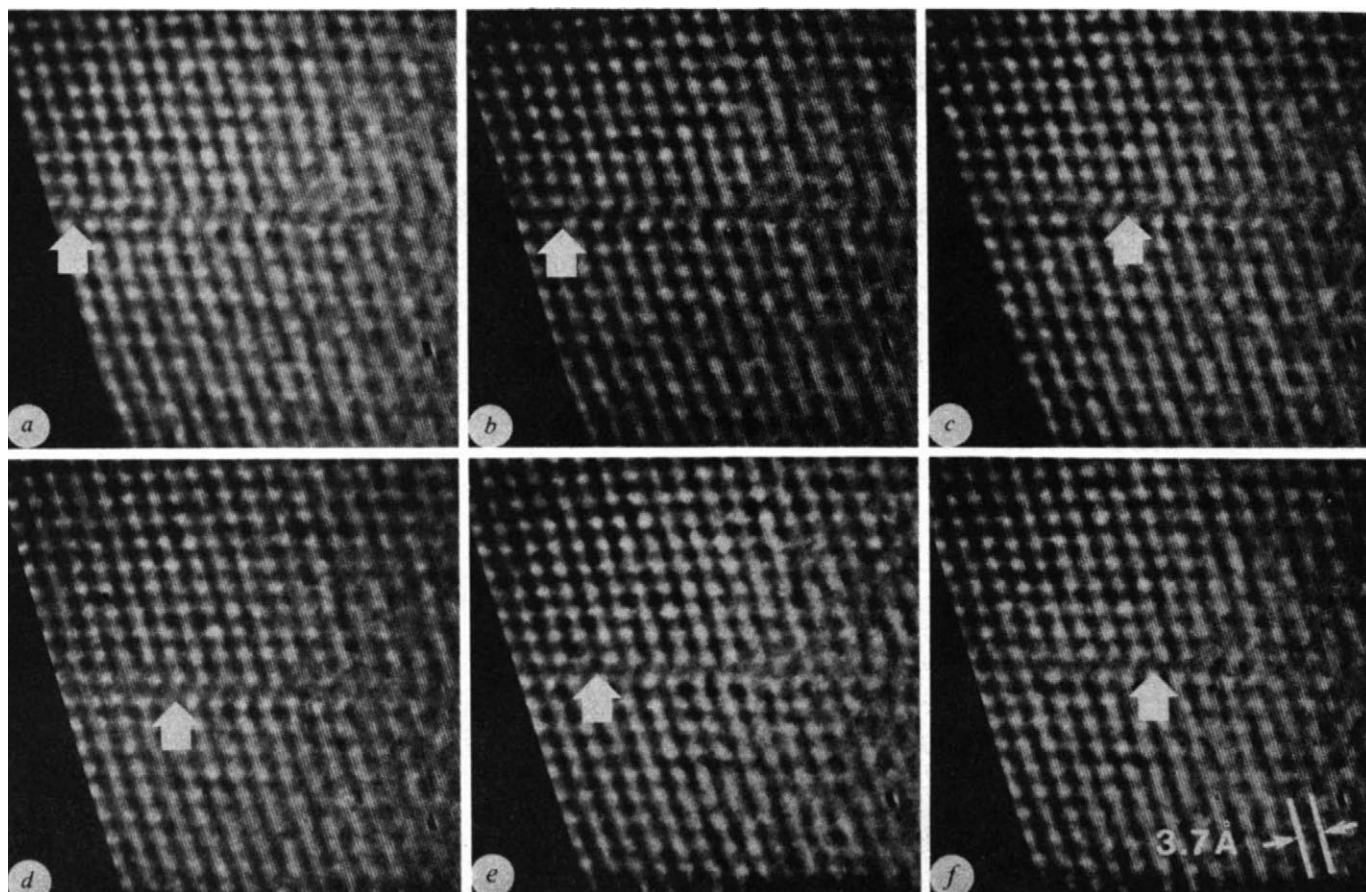


Fig. 3 Successive frames (0.02 s apart) showing the movement of a partial dislocation (arrowed), from left to right, along its {111} slip plane. As the dislocation moves, an intrinsic stacking fault is destroyed and the crystal is repaired.

system was closer to $3 \text{ \AA}$, that is the video-tape images are similar in resolution to those obtained photographically at 120 kV previously⁴. Bright spots in the image represent a projection of the CdTe pair of atomic columns, separated by $1.6 \text{ \AA}$ but unresolved (for further structural details see ref. 4). Image matching calculations show that the spots coincide either with the atomic columns ('white atoms') or with the open channels in the zincblende structure ('black atoms'), depending on the objective lens defocus, with the latter often yielding the highest contrast image. This condition can be recognized from the image appearance at stacking faults⁹⁻¹¹.

The specimen was imaged in the [110] zone axis orientation at 500 kV, with a current density at the specimen of $\sim 0.01 \text{ A mm}^{-2}$ and a beam divergence of 0.2 mrad. A large objective aperture (out to $1.2 \text{ \AA}$ d -spacing) was sometimes used, although its presence apparently did not affect the image. The image pick-up system incorporated an Image-Isocon low-light-level camera tube (EEV type P-8041), coupled fibre-optically to a transmission fluorescent screen at unit magnification⁸, the phosphor being P22 at 0.1 mg mm^{-2} coating density. At the phosphor screen the image magnification was ~ 1.3 million times, with a further 7.5 times to the television viewing monitor (that is a total magnification of ~ 10 million times). Recording was made with a standard video-tape system, and images for reproduction were photographed directly from the monitor during play-back.

There are several possible ways to examine and to display the images. First, the video-tape can be viewed continuously at normal (real-time) rate or in slow-motion, which is useful for qualitative observation of dynamic events. Second, the tape can be studied field-by-field (two interlaced 312.5 line fields = one 625 line frame) where successive fields are separated by 0.02 s. The noise in single fields can be reduced either by photographing the screen during continuous play-back using a longer photographic exposure time (for example, 0.5 s), or by

averaging several frames using a digital frame-store¹². The latter method permits the removal of non-uniform brightness due to the image system itself but, for the present specimen, the image contrast is so high that little benefit is gained by such image enhancement procedures. Calculation of the Fourier transform of the image (the diffractogram) is also possible using such a system: examples are shown in Fig. 1.

The CdTe specimen was prepared by mechanical polishing, followed by ion-beam milling, as in the previous work^{4,9}. The only difference here was the presence of a thin amorphous layer over much of the sample, possibly due to contamination in the microscope but thought more likely to arise from specimen changes in the time elapsed before examination.

Observations

The general macroscopic behaviour observed previously⁴ was reproduced in the present experiments: that is, after examination of a particular area for several minutes specimen degradation occurs more and more quickly with nucleation of new crystallites on the specimen surface and removal of material from the specimen edge. Most of the events of interest occurred before this process became too rapid to follow.

The original observations⁴ of image spots apparently jumping at the edge of the specimen were never repeated clearly. The impression gained was that particular edge spots would disappear and reappear in a random manner, but not that definite jumps from one site to another occurred. An example of the changes which can be seen between individual fields, fractions of a second apart, is shown in Fig. 2. Whether the difference arises from the presence of the amorphous film or from the different microscope environment and imaging conditions (electron energy) has not yet been established.

Conversely, many events were recorded which had not even been noticed previously. In particular, the defect distribution

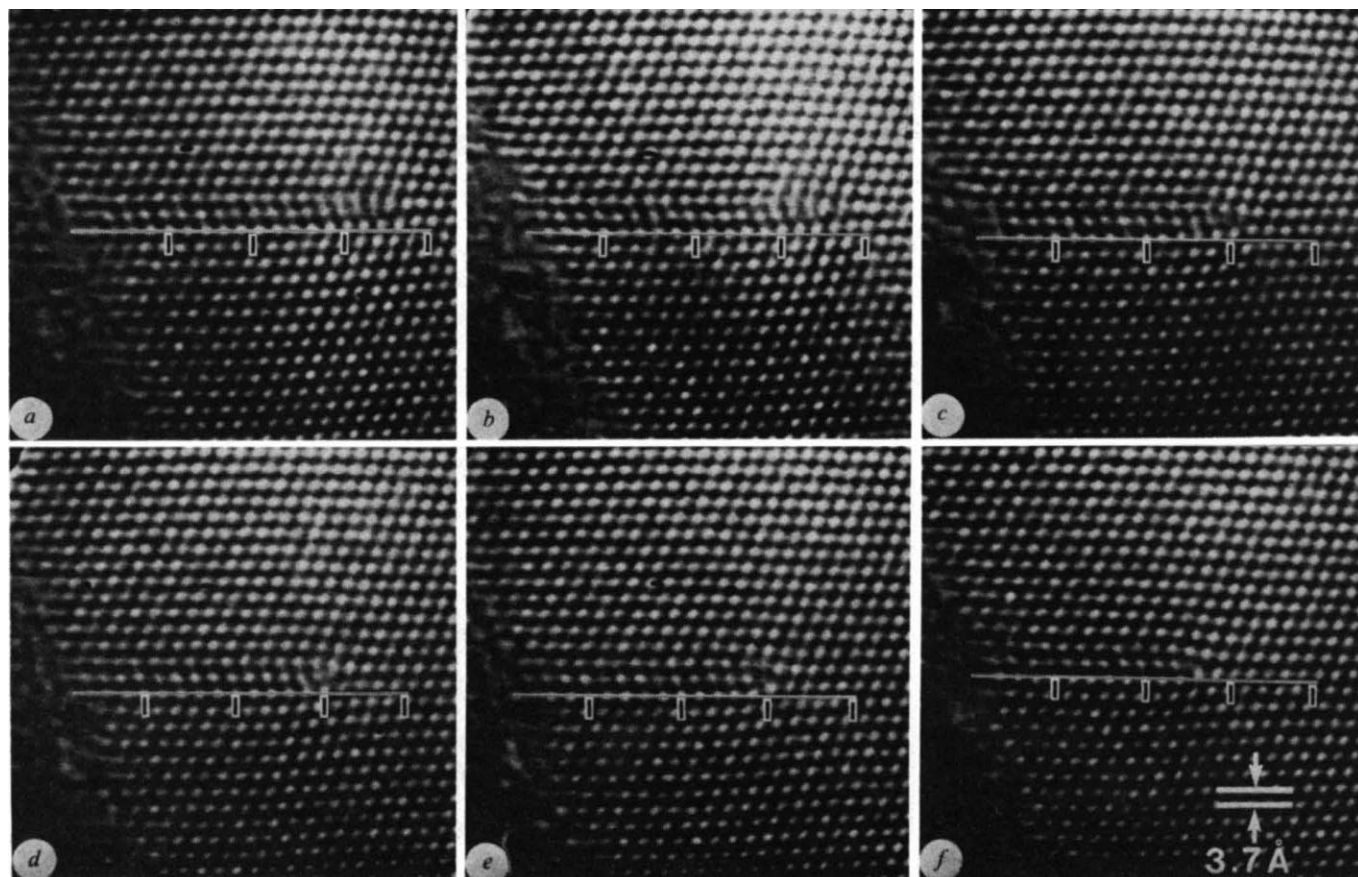


Fig. 4 Photographs showing the gradual disappearance of an extrinsic stacking fault, by removal of successive atomic columns at the interior end of the fault. The camera exposure times was 0.5 s and so each picture is a time-average of 25 frames. The time intervals and number of image spots (corresponding to CdTe atomic columns) in the fault are as follows: a, 00 s, 18 spots; b, 28 s, 17 spots; c, 48 s, 16 spots; d, 71 s, 15 or 16 spots; e, 85 s, 13 spots; f, 105 s, 12 spots. The reference markings correspond to every fifth image spot.

changed with time, with an overall tendency to defect annihilation. Thus the annealing behaviour of the material is being observed dynamically at lattice resolution. Amongst the events recorded were: (1) elimination of an intrinsic stacking fault on movement by slip of a Shockley partial dislocation; (2) extension of a dissociated dislocation by slip of one Shockley partial but not the other, creating an intrinsic fault as a consequence; (3) gradual disappearance of a $\{111\}$ planar defect, thought to be a vacancy loop, originally about 50 Å in diameter, over a period of 4 min, presumably by a diffusional mechanism; (4) disappearance of an unidentified 'cloud' associated with a perfect dislocation, about six planes in diameter over a period of ~ 80 s, leaving the perfect dislocation; (5) reduction in length of an extrinsic stacking fault (equivalent to an inserted $\{111\}$ plane) by one atomic column approximately every 20 s.

Events (1) and (5) have been chosen to illustrate the dramatic nature of these observations.

Individual image fields recorded during the annihilation of an intrinsic stacking fault by slip are shown in Fig. 3 (successive fields are separated by 0.02 s). The progress of the partial dislocation along the fault, recreating the perfect crystal, at an approximate rate of one $\{111\}$ plane (3.7 Å) every 0.04 s, can be seen. Note that in the continuous tape, and in the original observation, the fault seemed to disappear instantaneously—only by examining the tape one field at a time could the present description be made. The dislocation velocity thus is much slower than speed of sound in the solid.

Photographs taken during removal of an extrinsic fault are shown in Fig. 4. The extra $\{111\}$ plane disappears one CdTe column pair at a time, as demonstrated by the disappearance of the image spot at the end of the fault, on average every 20 s. Alternatively, it can be stated that the Frank partial dislocation terminating the fault is moving towards the crystal edge by a

diffusional climb mechanism. Atomic motion is thought to occur, backwards and forwards along the final column (or dislocation core), with loss of atoms when they reach the broad crystal surfaces and migrate away. The final image spot is noticeably unstable in position and in brightness as it gradually disappears. When it has gone, the atomic planes on either side close up to resume their perfect crystal positions. The continuous recording was essential here to establish that the fault was moving out (that is spots are lost from the interior) rather than the crystal edge moving in (that is, spots lost from the edge, which would also reduce the apparent length of the fault). Individual conventional images could, in principle, provide similar information but without resolving this issue.

Discussion and conclusions

From the microscopist's point of view, the direct observation and recording of atomic redistribution is a significant technological achievement. But this is not the only important aspect of the work. Many electrical devices utilize the electronic properties of semiconductors, relying mainly on highly perfect, single crystal forms. Moreover, in future, one expects thin film devices to be more prominent, especially if they can be made relatively defect-free. The present study relates directly to defect annihilation in such thin films at close to the atomic level, from which much can be deduced concerning the relevant mechanisms.

These data need to be extensively analysed, and this will be reported later. Some refinement of this work is needed to determine whether this approach is relevant or whether it represents a useful, though artificial, way of studying imperfections. For instance, we have yet to establish whether the 500-kV electrons (or the 120-kV electrons) are influencing the behaviour other than by providing local heating. The results

observed may be due to some unique property of CdTe or they may be observed in other materials provided that the appropriate specimen temperature is achieved⁴. More controlled experiments, involving known temperature changes and good vacuum conditions, are also needed.

We conclude that it is possible to record dynamic events at close to atomic resolution in CdTe, a semiconductor material. Moreover, some of the microstructural changes observed can be interpreted directly in terms of atomic movement. This establishes the possibilities for further, more detailed and more controlled experiments in future on this and similar materials.

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F-actin is a helix with a random variable twist

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Image analysis of isolated F-actin filaments shows that the actin helix can be described by a constant rise per subunit but a considerably variable and randomized twist (number of units per turn). The ability of actin subunits to rotate through angles of the order of 10° from their helically ideal positions helps to explain actin's capacity to form many different polymorphic structures.

IN both muscle and non-muscle systems, the geometry of the F-actin helix is crucial to determining both sterically and stoichiometrically how actin binds to other proteins¹. We have been studying the structure of F-actin as part of a larger research effort aimed at understanding how cells can use a single highly conserved protein, actin, in a large number of different structures.

Several observations suggest that the structure of F-actin is characterized by a significant amount of disorder. Hanson's observations of negatively stained F-actin filaments in the electron microscope showed that the cross-over points (generated by the 'crossing-over' of the two long-pitch actin helices) were quite variable². However, she was unable to exclude the possibility that this variation was a result of specimen preparation. X-ray diffraction from muscle, in physiological conditions, led Huxley to suggest that "actin behaved like a twin strand of beads, which could be twisted easily but not stretched"³. Our own electron microscopic observations, using computed image analysis, indicate that specimen preparation is not responsible for variable cross-overs, and suggest a model for this disorder which yields quantitative statements about the magnitude of the displacements of subunits away from their helically ideal positions. The model, which has a randomly varying twist between subunits, is important in understanding actin bundle formation and the attachment of myosin cross-bridges to muscle thin filaments.

Observations of isolated F-actin filaments

Values for the twist of actin (the number of subunits per turn of actin's left-handed 59 Å pitch helix, see Fig. 1) have been reported. The twist of the actin helix determines how various

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- subunits are positioned with respect to each other, and consequently is important in understanding how an aggregate of filaments may be assembled. These values have been determined for aggregates of actin such as Mg²⁺ paracrystals⁴, cross-bridged bundles⁵ and muscle fibres⁶ rather than isolated filaments. Most values have fallen in the range 2.154–2.167, which corresponds to an angular separation between subunits in the helix of 167.14–166.15°. We have measured units per turn for isolated filaments using computed transforms. Electron micrographs of negatively stained filaments were digitized, Fourier transforms were computed, layer line spacings were measured, and the number of units per turn calculated. Computed transforms provided information on single filaments that in practice would have been difficult to obtain with optical diffraction patterns for the following reasons: the computed transforms minimize the contribution from the mask used to separate a particle from a field; eliminate effects due to optical inhomogeneities; allow for easy manipulation of threshold and contrast in the display of the resulting transform; and provide phase information. Figure 1 compares the twists calculated for several Mg²⁺ paracrystals with that for a number of isolated single filaments of actin from chicken skeletal muscle.

These histograms clearly show two features; one is that the mean is shifted from 2.166 for the paracrystals to 2.159 units per turn for the single filaments. This shift corresponds to an average untwisting of the actin helix of 0.5° per subunit. The second feature is that the variance of the single filament population is almost 10 times greater than that of the paracrystal population. We have data for three other species of actin (sea urchin cytoplasmic, scallop adductor muscle and *Acanthamoeba* cytoplasmic; data not shown) involving both single filaments and paracrystals which display similar features. In all cases, the

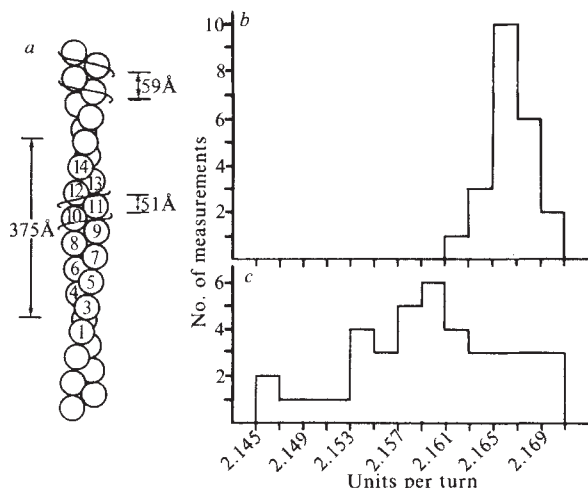


Fig. 1 The actin helical geometry and our measurements of it for aggregates and single filaments. *a*, The 'ping-pong ball' model is designed to show the filament geometry (relative subunit positions) and not to provide any information about subunit shape or filament appearance. Three types of helices which link the subunits are illustrated: the two long-pitch helices (~ 750 Å in pitch) which 'cross over' at ~ 375 Å, as well as the single 51 Å pitch helix, are right-handed; the 59 Å pitch helix, often called the actin genetic helix, is left-handed. *b*, *c*, Measurements of units per turn were made for chicken skeletal muscle actin. F-actin from adult White Rock chicken pectoralis muscle was purified by the procedure of Spudis and Watt²⁰ using an acetone powder. The final polymerization was initiated by adding MgCl_2 to 2 mM and KCl to 10 mM to a solution of G-actin in 0.2 mM ATP, 2 mM Tris (pH 8.2), 0.5 mM dithiothreitol and 0.2 mM CaCl_2 . All specimens were negatively stained with 1% uranyl acetate. Determinations of units per turn were made with computed transforms of the electron micrographs, using at least three layer lines in all cases (the first, second and sixth for paracrystals; the first, sixth and seventh for single filaments). *a*, Mg^{2+} paracrystals were formed by raising the MgCl_2 concentration to 50 mM. For the 22 paracrystals measured, the average number of units per turn was 2.166 (0.002 = s.d.). *b*, Isolated single filaments were examined with the same method. The average units per turn was 2.159 for the 39 filaments shown (0.006 = s.d.). The almost 10-fold increase in the variance of the units per turn for the single filaments could not be explained by a corresponding increase in the error associated with determining this parameter for single filaments. Although the lengths examined for both paracrystals and single filaments were comparable ($\sim 3,000$ Å for both), there were many filaments contained in every paracrystal area measured. However, this increase in averaging could not explain the observed difference in spread, as single filaments masked off from within a given paracrystal behaved much more like the aggregate paracrystal than an isolated single filament.

variance of the single filaments is comparable with that shown for chicken skeletal actin. Paracrystals from different sources of actin have a measured symmetry which is not significantly different from that of chicken skeletal actin at 2.166 units per turn. The means for populations of single filaments, however, did differ for different actins: scallop, 2.154 ± 0.002 (s.e.m.); sea urchin coelomocyte, 2.167 ± 0.002 .

Our data show that the twist of actin can be altered by its inclusion in paracrystals. Changes of this magnitude have previously been noted for thin filaments⁴, as well as for actin-containing bundles in *Limulus* sperm⁷. Further, there is a large increase in the 'spread' of the single filament measurements relative to that of the paracrystals. The interesting question is whether the variations in twist as seen in Fig. 1 are a real property of isolated filaments.

First, we could exclude the possibility that the features of the single filament histogram are merely the result of gross damage resulting from specimen preparation, because our measurements of phase residuals across layer lines (an indication of asymmetry between the two sides of a filament) are $\sim 10^\circ$, better than the published residuals of filaments within Mg^{2+} paracrystals^{8,9}. Second, because the same procedure and algorithm (computer program) were used to locate layer lines for paracrystals and for single filaments, the difference was not in the method but in the diffraction patterns themselves.

As the variance in estimates of units per turn for a single filament (derived from different layer lines) was roughly equal

to the variance in units per turn for the population of particles, we could rule out a model in which each filament is a perfect helix but different filaments have different helical parameters. If this model were correct the variance in units per turn for each filament would be much less than that of the population. Instead, our results showed that the variance was a result of deviations in the measured positions of the various layer lines from those expected for a perfect helix. We will now present a particular model for these departures from helical symmetry and then show that it accounts for the variation in units per turn, the observed deviations of layer line positions, as well as previous observations about disorder in F-actin. This model leads to quantitative estimates of the magnitude of this disorder.

A model for actin having variable twist

All X-ray studies of muscle and actin gels^{6,10} have shown well-defined reflections for the 59 and 51 Å one-start helices, as well as the 27.3 Å rise per subunit. In fact, diffraction from oriented F-actin gels extends out to the fourth order of the meridional at ~ 7 Å, with many reflections in between corresponding to one-start helices. The sharpness of the true meridional reflections seen in these X-ray patterns is one of the main reasons that we believe that actin subunits do not have any substantial component of disorder in the axial direction (thus, the disorder must be entirely azimuthal in nature). To understand the model of azimuthal disorder, first consider a perfectly helical actin filament. In such a filament each subunit is related to the next by a rotation of $\sim 167^\circ$ (the exact rotation specifies the twist) around the axis and a rise or translation of 27.3 Å along the axis. In the variable twist model we propose, the axial rise is still constant at 27.3 Å, but the rotation between subunits varies and this variation is cumulative. For the variable twist filament, let the random angular deviations from 167° be δ_2 for subunit 2, δ_3 for subunit 3 and so on. If the position of subunit 1 is 0° , 0 Å, then, for subunit 2, $\theta = 167^\circ + \delta_2$, $z = 27.3$ Å; for subunit 3, $\theta = 2 \times 167^\circ + \delta_2 + \delta_3$, $z = 2 \times 27.3 =$

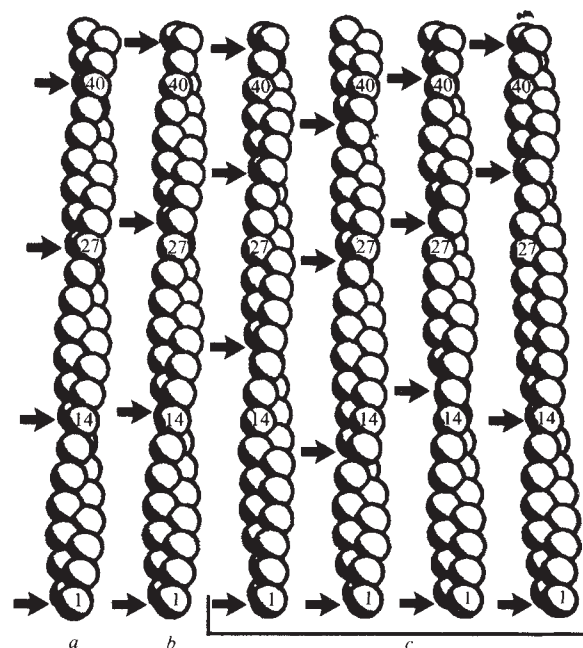


Fig. 2 The model shows how 'twist' affects the appearance of the actin filament. All six filaments are drawn with their first subunit at the same angular position. The axial rise per subunit is also constant in all filaments. *a*, This filament was generated with 13 subunits per cross-over (2.167 units per turn). Cross-overs will therefore appear every 354 Å for a 27.3 Å rise per subunit. *b*, This filament contains 14 subunits per cross-over (2.154 units per turn) and its cross-overs will appear regularly at 382 Å. *c*, These four filaments were generated with rotational disorder. The r.m.s. fluctuation per subunit in deviations from the expected angular position is 10° . Because the deviations are cumulative, the cross-over spacings now appear to be significantly randomized.

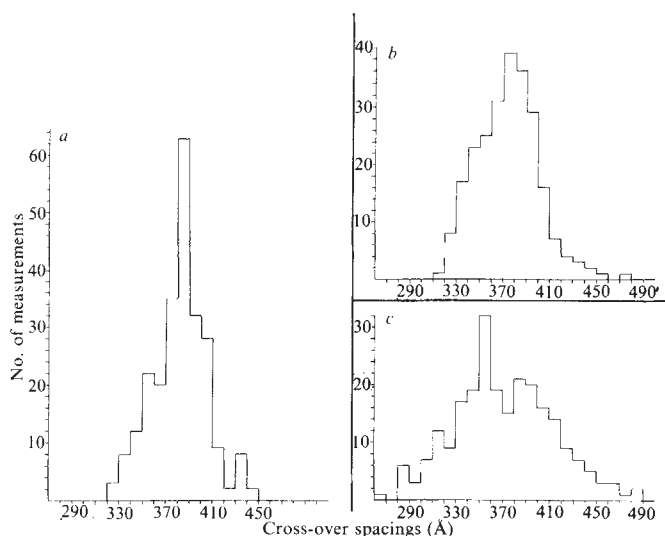


Fig. 3 The flexibility in 'twist' of the actin helix will lead to a large variation in the observed cross-over spacing, as illustrated in Fig. 2. Actual measurements of this distance may be compared with the predictions of our model. *a*, We reproduce here the data of Hanson² on negatively stained scallop muscle thin filaments. These distances were determined by measuring the spacing between from two to six cross-over points along a filament, with measurements most frequently being made upon three cross-overs. Thus, each entry in the histogram ($N=243$) already represents some averaging over several cross-over repeats. For comparison, note that the amount of this averaging is less than that used by us with Fourier methods (in Fig. 1, each entry in our histogram for single filaments corresponds to an average of about eight cross-overs). The average single spacing in Hanson's data is 382 Å (23.3 = s.d.). *b*, A computer model was generated with a r.m.s. angular excursion of 6° per subunit. Each entry ($N=243$) represents an average determined from the distance between three cross-over points. The standard deviation is 26.7 Å, which is not significantly different from that of Hanson's distribution. *c*, The same computer model and method was used as in *b*, but with a r.m.s. angular deviation per subunit of 10°. The standard deviation of this distribution ($N=241$) is 42.4 Å. We believe that the 6 r.m.s. value represents the lowest limit for the flexibility of the actin twist, and that the 10° value fits other data better (see text).

54.6 Å, and so on. The variability can be characterized by the r.m.s. value of all the δ_i s which we will call δ :

$$\delta = \left(\frac{1}{N} \sum_{i=1}^N \delta_i^2 \right)^{1/2}$$

From a value of δ we can then predict the deviations from the expected values for the angular positions of subunits; for example, if $\delta = 10^\circ$, we would expect to find: for subunit 2, $\theta = 167 \pm 10^\circ$; for subunit 3, $\theta = 2 \times 167^\circ \pm \sqrt{2 \times 10^\circ} = 334 \pm 14^\circ$, and so on.

A model incorporating these displacements of subunits is shown in Fig. 2c. A gaussian distribution for δ was chosen for computational convenience only. There is no evidence that the actual distribution of angular deviations in actin is also gaussian.

It was necessary to allow the angular disorder per subunit to be $\sim 10^\circ$ so as not just to mimic the distribution in units per turn of single filaments, but to match several of the features found in single filament transforms (see Fig. 4). This seemed rather large and raised the question of whether it corresponded to a physically reasonable deformation of the structure. We have compared the deformation due to such an angular motion with the deformation involved in the bending of actin filaments in solution. Quantitative observations of actin flexing¹¹⁻¹⁴ can be extrapolated back to the displacements between subunits. Typical displacements between alternate subunits at 20°C would be ~ 1.5 Å. The angular motions we describe would lead to displacements of ~ 2.5 Å between these same subunits at a reasonable radius of contact. Our comparison with bending shows that the deformations overlap considerably in magnitude with deformations which have already been observed for F-actin. Further, both forms of deformation (flexing and angular motions of subunits) reflect the same underlying physical phenomenon. Locally, subunit positions are well correlated

with each other, but this correlation falls off the further away one goes from a given subunit.

In our model some relationships between subunits are relatively fixed (the axial rise) and some have a certain amount of freedom (angular rotation). For a filament having protomers made of several thousand atoms, the freedom of subunits to bend with respect to each other must represent a sum over many different internal modes of freedom. Thus, although the observed flexing of F-actin may be the result of the ability of actin subunits to rotate with respect to each other, this question requires much higher resolution data on F-actin. We have chosen to represent the subunits in our figures as rigid, with the deformation occurring between them, for convenience only. It is more probable that the interface between subunits has the same degree of elasticity as the interior of the subunit itself. Given new data on the structure of G-actin obtained by X-ray diffraction¹⁵ and image analysis¹⁶, which show two domains separated by a cleft, it is interesting to speculate that there may be an internal hinge within the molecule which contributes substantially to the observed flexibility.

Predictions of the model

The first prediction concerns the amount of variability between cross-over points within a filament. For example, for a perfectly helical actin having 2.154 units per turn or 167.1° between subunits, cross-overs will appear every 382 Å (see Fig. 2b). For an actin filament having 2.167 units per turn or 166.1° between

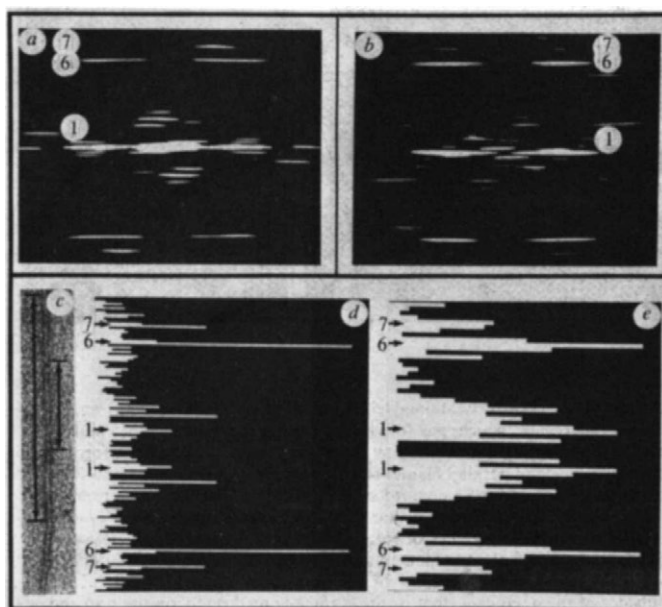


Fig. 4 Fourier transforms were computed for over 150 isolated actin filaments. *a*, *b* Show typical examples of these transforms, where each was computed from a stretch of filament containing ~ 100 subunits. The layer line numbering scheme shown for each of these transforms corresponds to an actin helix having 13 subunits in 6 turns of the 59 Å pitch helix. The sixth layer line corresponds to this 59 Å helix, the seventh layer line corresponds to the 51 Å pitch helix, while the first layer line corresponds to the two 750 Å pitch helices which cross over at ~ 375 Å. These three helices are illustrated in Fig. 1. The most significant feature was that the sixth and seventh layer lines were generally well defined, while the first layer line varied widely between transforms, often diffuse or split, and sometimes almost completely absent. A section of chicken actin filament is shown in *c*. The long stretch marked on the left of the filament contains ~ 90 subunits, and its transform is shown in *b*. To illustrate the relative layer line intensities graphically, we have projected all the intensities in *b* (excluding the equator) on to the meridian of the transform. This projection is shown in *d*. The first layer line can be seen to be quite weak, with an intensity less than that of the seventh. The unusually strong peak at about the second layer line position is not a typical feature of the single filament transforms, but is rather representative of the fluctuations in various layer line intensities which occur from transform to transform. The short stretch marked on the right side of the filament in *c* contains ~ 35 subunits. This section was also transformed (transform not shown) and projected exactly as for *d* and the projection is shown in *e*. The first layer line relative intensity increases for the shorter segment, almost to the height of the sixth layer line.

subunits, cross-overs will occur every 354 Å (Fig. 2a). For filaments having a variable twist, the cross-over distance will itself be variable (as Hanson observed) and the amount of variability gives one measure of δ (Fig. 2c).

The second prediction concerns how the transform of the filaments will be affected by the variable twist. A rigorous treatment of the problem is presented elsewhere¹⁷. One prediction is that, with increase in the length of actin from which the

diffraction pattern is produced, the intensity of the first layer line will fall relative to the sixth and seventh layer lines.

Variable cross-over spacings

Figure 3 compares Hanson's observations, based on the measurement of several hundred cross-overs in isolated thin filaments (actin and tropomyosin), with our model predictions. Hanson found a large variation in cross-over spacings, and the spread in her data could not be explained by measurement error. Thus the possibility remained that there was a variation in the helix along an individual filament. She then noted: "If the variability in the axial period of the cross-overs reflects a genuine variability in structure, present in the filaments before preparing them for examination in the microscope, this would be of considerable interest."

As Hanson's measurements were not made on single cross-over distances, but rather on two to six cross-overs, her data, shown in Fig. 3a, already represent a certain amount of averaging for every entry in the histogram. We therefore imposed a similar averaging on our computer model to generate comparable histograms as a function of δ , the r.m.s. angular fluctuation. Every entry in Fig. 3b and c represents the average distance per cross-over as measured from three cross-overs. By using this method, we found that a value of $\delta = 6^\circ$ fits Hanson's data rather well, while the value of $\delta = 10^\circ$, obtained from our other observations, produces a distribution almost twice as wide as Hanson's. However, we feel that there is not necessarily any irreconcilable discrepancy (the difference cannot be attributed to the presence of tropomyosin, because Hanson measured the same variance for pure actin filaments). It is quite likely that the average number of cross-overs measured by Hanson was greater than three, which she stated was the most frequent measurement. By increasing the average number of cross-overs included within each measurement, the averaging is increased and the distribution would be narrowed. Further, because Hanson's 243 measurements were extracted from observations of 2,000 filaments, it may be that the more periodic segments of actin were selected, which would force her histogram (shown in Fig. 3a) to appear narrower than the distribution generated by a truly unbiased selection of data.

Coherence lengths within the filament

The second prediction of the model concerns the difference in coherence lengths for different helices within the F-actin filament. The effect of this disorder on any given layer line will only depend on the Bessel order n of that layer line, and not on the pitch of the corresponding helix. The sixth and seventh actin layer lines ($n = \pm 1$) are related to the short pitch single helices which link every adjacent subunit, while the first actin layer line ($n = 2$) is related to the two long pitch helices, each of which links alternate subunits. Because of the cumulative nature of the angular disorder, it can be shown that correlation within a helix will die off faster if that helix relates subunits which are further separated from each other. Taking $\delta = 10^\circ$, a complete analytical treatment shows that all one-start helices (relating adjacent subunits) will have a fundamental correlation length containing 125 subunits, while the two-start helices (relating alternate subunits) will have a correlation length only one-quarter of this, or ~ 31 subunits. The effect predicted by our model is that for short stretches (containing of the order of 30 subunits) both the one-start and the two-start helices should be fairly coherent over this length, that is, all subunits in the segment will be contributing coherently to the first and sixth layer line intensities. However, when one transforms longer lengths (containing of the order of 125 subunits), one expects that all subunits will be contributing coherently to the sixth layer line intensity, but that many subunits will be contributing incoherently to the first layer line intensity. Thus, the relative peak intensity of the first layer line will fall on the

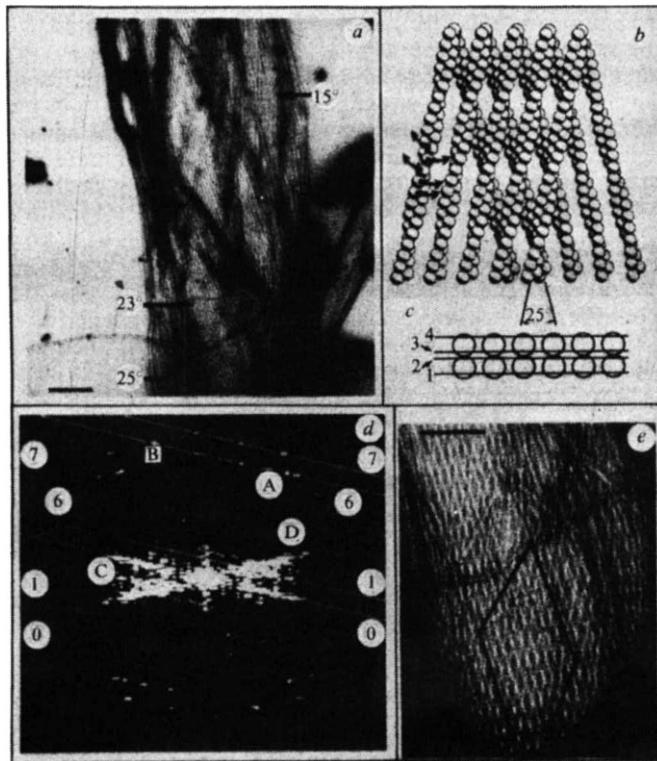


Fig. 5 *a*, A negatively stained electron micrograph of an actin angle-layered aggregate, which was formed by raising the F-actin to 25 mM in MgCl_2 . *b*, This diagram shows how the angle-layered aggregate is formed from two sheets of parallel filaments which are rotated with respect to each other, as described by Yamamoto *et al.*¹⁸. However, unlike their observations, one can clearly see in *a* that the angle of rotation is variable, with regions shown having angles of rotation of 15° , 23° and 25° . *c*, These two overlapping sheets of actin filaments may be further decomposed into four surfaces: 1 and 4 are the sides of the actin filaments which are on the outsides of the aggregate (that is, the surface lying directly on the grid and the surface furthest from the grid), while 2 and 3 are the inside surfaces of the actin filaments in this structure, the only interactions are between filaments in different sheets (that is, between surfaces 2 and 3). Thus this structure is intermediate between isolated filaments and Mg^{2+} paracrystals. The area enclosed within the dotted line in *a* has been magnified in *e* (Scale bar, 1,000 Å). *d*, The computed transform of the region shown boxed in *e*. As the angle-layered aggregate may be thought of to a first approximation as the linear superposition of the two sheets shown in *b*, the corresponding transform of this structure may also be thought of as the linear sum of the transforms of each of the two sheets. Thus, we can index *d* with the two sets of actin layer lines shown. The layer lines corresponding to the top sheet are shown with solid lines, and the layer lines corresponding to the filaments in the bottom sheet are shown with broken lines. The two sets of layer lines are rotated with respect to each other by 25° , which is the angle of rotation of one set of filaments with respect to the other. A and B correspond to the reflections lying along the sixth layer line of the top sheet of filaments. It can be seen that B is a very well defined and sharp spot, while A is much more of a streak containing four spots extending along the layer line. Similarly, the spots CD lie along the first layer line of the bottom sheet. Spot C is relatively sharp, while D is weak and diffuse. This combination of sharp and diffuse reflections is generated by the combination of order (the same specific interactions that points of contact between filaments in different sheets) and disorder (variable cross-over spacings resulting from the angular disorder). A full description of the transform requires a model containing disorder as well as appropriate filament shape²¹.

average with respect to that of the sixth layer line as the length of filament increases. Figure 4 demonstrates how our single filament data confirmed this prediction of the model. Because the prediction is so specific, dealing with the relative behaviour of different component helices as a function of length, we feel that its confirmation effectively excludes the possibility of other kinds of disorder (such as local bending of filaments) being responsible for our observations.

We can extend this discussion to X-ray results in physiological conditions. For large numbers of subunits, the peak intensity of any given layer line will be weighted by $(1/n)^2$, where n is the principal Bessel order on that layer line. Similarly, the width of the layer line will be proportional to n^2 . These results hold for any degree of cumulative disorder, as long as one is dealing with lengths greater than the longest correlation length. In contrast to the well defined first layer line seen in the electron microscopic investigations of actin aggregates, the situation in X-ray diagrams of muscle at rest (where there are relatively free thin filaments) was summed up by Huxley and Brown thus: "... [the recorded pattern] does show that an arrangement of actin monomers capable of giving strong and well-ordered reflections at 59 Å and outwards, does not necessarily give appreciable diffracted intensity on a layer line in the 350–400 Å region [the 1st layer line]"⁶. We believe that the lack of a sharp first actin layer line when diffracting from non-interacting filaments is a consequence of cumulative angular disorder: the peak intensity of this layer line will fall by a factor of four with respect to the sixth and seventh layer lines, while its width will increase by a corresponding factor of four, further helping to bury the reflection in the diffuse continuum of background scatter.

Because both X-ray patterns from muscle and Hanson's measurements have involved thin filaments and not just pure F-actin, they suggest that tropomyosin's presence does not qualitatively change the motions of the actin subunits. As the presence of tropomyosin contributes less than 20% increased rigidity to an actin filament¹³, the intrinsic flexibility of tropomyosin is large enough for the deviations due to the thermal bending of tropomyosin in solution to be less than those of a tropomyosin molecule having to follow the disordered actin positions at a radius of 25 Å.

Angle-layered aggregates

Angle-layered aggregates of F-actin, formed at Mg^{2+} concentrations lower than that used for paracrystals, consist of two sheets of parallel filaments which have been rotated with respect to each other¹⁸. Because the spacing between filaments in a single sheet ranges from 95 to 160 Å, depending on the angle of rotation (see Fig. 5), most of the subunits in these aggregates will not interact laterally with other filaments. The only interactions will occur between the sheets, with subunits from one sheet interacting with subunits from the other sheet. Thus, we would expect the transform of this structure to display features characteristic of both order and disorder. We have found this to be the case (Fig. 5). The variable cross-overs present within free filaments are incorporated into the angle-layered aggregates, leading to a variation in the inter-filament spacing within each of the sheets. The interaction between the sheets is specific (it occurs near the cross-over points), and filaments are shifted axially until subunits are aligned. Because the disorder present

within single filaments is 'locked-in' to the angle-layered aggregate structure at the time it is formed, it is implausible that this disorder is a consequence of the preparation for electron microscopy. Because both the features characteristic of order (related to the specific Mg^{2+} inter-filament bond) as well as the features characteristic of disorder (related to the intra-filament randomly variable twist) appear simultaneously, it is much more reasonable to believe that the angle-layered aggregates provide an interesting 'transition' between free filaments and tightly packed bundles.

All previous image analyses of F-actin have been performed on aggregates of filaments. It is quite likely that in structures such as paracrystals and cross-bridged bundles, most subunits are interacting with each other or with a cross-bridging protein. However, these structures have been considered very useful because they allow for signal averaging over many filaments, each of which is presumed to be an ideal helix. Our model of flexible twist, as well as image analysis of angle-layered aggregates, suggests instead that within these aggregates particular forms of deformation of the actin helix are stabilized by the inter-filament packing. Because of the intrinsic variability in twist, the exact conformation of the actin helix in assemblies will not be governed by intra-filament bonds but rather by inter-filament interactions. We predict that actin filaments in such bundles are not helical. This important point is illustrated in the structure of needles.

An analysis of cross-bridged actin bundles (needles)¹ has provided some insight into how actin filaments, which have no hexagonal symmetry, may be packed into hexagonal bundles. Within these bundles, every actin filament will interact with six surrounding filaments, that is, bonds will be made at 60° increments around the filament. As the 'ideal' subunit angular positions do not fall on multiples of 60°, it was postulated that a cross-bridging protein could be deformed in order to compensate for the fact that subunits could be as much as 6.5° away from hexagonal positions. This type of deformation was necessary to explain the observed pattern of cross-bridging. It must be more than coincidence that this amount of angular excursion is well within the range of motion which actin subunits may travel through. As long as subunits are in axial register (maintained by the constant axial rise per subunit), actin subunits can rotate through significant angles to form equivalent interactions with other filaments along their length. Because an actin filament which had fixed subunit angular positions could not make the bonds necessary to form any of the many regular aggregates of actin observed, we suggest that the ability of actin subunits to rotate may be crucial to actin's ability to form many polymorphic structures. Further, the angular motions of actin subunits may have a role in the function of muscle. It is geometrically impossible for thick and thin filaments, which possess different helical symmetries, to make equivalent interactions throughout the muscle lattice. It has become a working assumption that myosin heads rotate through significant angles to bring them into a better juxtaposition with actin subunits¹⁹. The motions of actin described here are probably equally necessary for the actomyosin interaction.

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A covalent adduct between the uracil ring and the active site of an aminoacyl tRNA synthetase

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A covalent adduct of an aminoacyl tRNA synthetase and uracil nucleoside has been isolated. The enzyme adduct is catalytically inactive; one nucleoside is bound per catalytic site. The release of uridine restores enzyme activity. The nucleoside attaches to a protein segment required for tRNA interaction. The findings add support to concepts of a covalent component for some protein–nucleic acid complexes.

AMINOACYL tRNA synthetases establish rules of the genetic code by matching each amino acid with exact trinucleotide sequences (anticodons) within specific tRNA molecules^{1–4}. Selection of correct tRNAs for each amino acid occurs by unknown mechanisms and may or may not be influenced by anticodons¹. Better understood is the overall mode of synthetase binding which is along and around the 'inside' of the L-shaped three-dimensional tRNA structure^{5,6}. Over 150 cytoplasmic tRNAs sequenced to date contain uridine (or 4-thiouridine) at position 8 which lies on the inside of the vertex of the L formed by the folded nucleic acid^{7,8}. Synthetases have long been known to make contact with this general area of the structure and some data suggest that synthetases transiently bind covalently to the uracil ring at position 8 in tRNAs^{2,9,10}.

Evidence for this^{9–11} is synthetase-catalysed H-5 exchange at uridine 8. Based on numerous investigations, the mechanism for H-5 exchange is nucleophilic attack by the enzyme on C-6 of the uracil ring to produce a Michael adduct; reversal of the reaction can give exchange (at C-5) with solvent-based hydrogen atoms^{2,9–20}. Subsequent studies showed that synthetases have a reactive 'uridine site' which forms a transient Michael adduct with mononucleoside uridine¹¹. This site is presumably the same one which attacks uridine 8 in tRNA.

There have previously been no data which establish the uridine site as necessary for catalytic activity or even determine its stoichiometric ratio with active centres on the enzyme. With another system—thymidylate synthetase—Michael addition to the uracil ring of dUMP is a key step on the pathway to thymidylate^{12,13,18,19,21–30}. The site responsible for Michael addition can be covalently labelled with a dUMP analogue at a ratio of one per active centre to produce inactive enzyme^{12,13,21–30}. Michael adducts have similarly been implicated in reactions catalysed by dUMP and by dCMP hydroxymethylase^{14,20}. Nucleic acid-modifying enzymes such as pseudouridylate synthetase and uracil-*N*-glycosidase, which act on tRNA and DNA, respectively, may also use Michael adducts as key catalytic intermediates³⁰.

If it is essential for enzyme function, covalent labelling of the uridine site of a synthetase should inactivate tRNA aminoacylation activity and should be stoichiometric with the number of active centres. Success on these counts would establish functional significance for the Michael addition site in a system of enzymes involved in protein–nucleic acid interactions.

Rationale for covalent labelling

Although there is much evidence for transient Michael adduct formation between aminoacyl tRNA synthetases and the uracil ring^{2,9–11}, a stable nucleoside–enzyme adduct has not been captured. For this purpose 5-bromouridine was used as a site-directed reagent because the electron-withdrawing power of halogen increases the susceptibility of C-6 to nucleophilic attack; additionally, model studies have shown that Michael

adducts of halogenated uracil compounds are capable of further rearrangements which produce stable complexes^{31–38}. The strategy failed previously, however, because all five synthetases tested catalysed deribosylation of 5-bromouridine to give bromouracil, ribose and free enzyme¹¹. While such results further demonstrate the existence of the uridine site and transient Michael addition, the desired stable adduct was not formed.

Two parameters were subsequently discovered to be crucial for stable covalent binding: the omission of sulphhydryl reducing agent and the use of 10-fold higher 5-bromouridine concentrations than used earlier (for example, 50 mM rather than 5 mM). Figure 1 depicts a rationale for covalent labelling. Nucleophilic attack at C-6 produces a transient Michael adduct which, predominantly, either dissociates by reversal or undergoes deribosylation to generate free enzyme. However, the occasional vertical pathway leads to a dead-end complex which, over time, accumulates until no free enzyme remains. High bromouridine concentrations drive the enzyme through many cycles and increase the yield of dead-end product. Reduced sulphhydryl compounds attack the irreversible complex and release free synthetase and uridine (data shown below).

The success of this strategy for covalent labelling is demonstrated below. While various schemes are known^{31–38}, a simple possibility for rearrangement of the Michael adduct, consistent with data presented here, is attack at C-5 by a secondary enzyme nucleophile to displace bromide and give enzyme linked through C-5 and C-6 to nucleoside³⁴ (see also ref. 39). Treatment of this adduct with reduced sulphhydryl compound generates free enzyme concomitant with the release of uridine³⁴.

Activity is lost in a first-order process

Figure 2a shows the time-dependent loss of aminoacylation activity of *Escherichia coli* Ala-tRNA synthetase, after exposure of the enzyme for various times at 24 °C to 80 mM bromouridine, in the absence of sulphhydryl reducing agents such as mercaptoethanol or dithiothreitol (DTT). The experi-

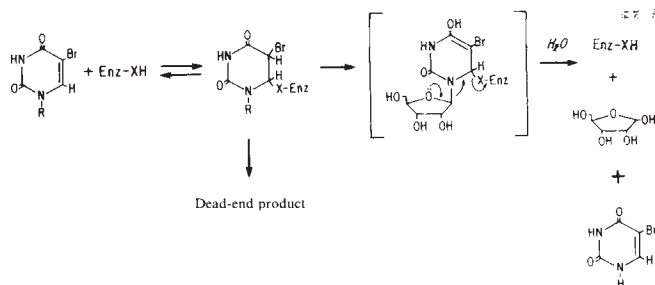


Fig. 1 Scheme for enzyme interaction with 5-bromouridine. Michael addition between an enzyme nucleophile XH and 5-bromouridine leads to deribosylation¹¹ and occasional rearrangement of the Michael adduct to give a dead-end complex described further in the text.

Table 1 Stoichiometry of [^{14}C]bromouridine binding

	Nucleoside bound per catalytic site
Ala-tRNA synthetase + [^{14}C]BUrd	0.86
Ala-tRNA synthetase + [^{14}C]Urd	0
Ala-tRNA synthetase + 10 mM DTT + [^{14}C]BUrd	0
Ala-tRNA synthetase + [^{14}C]BUrd at $t_{1/2}$ *	0.59
Phosphorylase + [^{14}C]BUrd	0
Ala-tRNA synthetase + [^{14}C]BUrd followed by SDS	0.95

All values are averages of two to five determinations. Nonspecific binding of nucleoside amounted to 0.2 mol per mol and was subtracted from all values; this value (0.2) is obtained when Ala-tRNA synthetase and uridine or phosphorylase and bromouridine are incubated together. For the experiment which included a SDS step the enzyme-bromouridine mixture was incubated at 90 °C for 2 min in 3% SDS before chromatography on an SDS-equilibrated column. The result of a comparable incubation with phosphorylase and bromouridine was considered background. Rabbit muscle phosphorylase was from Sigma. BUrd, 5-bromouridine; Urd, uridine.

* Ala-tRNA synthetase was incubated with [^{14}C]BUrd long enough to produce 50% inactivation.

ment involved exposing enzyme for various times to 5-bromouridine and then diluting out excess halopyrimidine and assaying enzyme activity. Enzyme incubated for 125 min in the absence of bromouridine retains full activity. Brief exposure (<1 min) to 80 mM bromouridine has no effect on activity, whereas exposure for 125 min almost totally inactivates the enzyme.

Figure 2b shows that activity loss of Ala-tRNA synthetase on exposure to bromouridine follows first order kinetics from 100% to less than 10% activity. At 80 mM bromouridine, the rate constant for inactivation at 24 °C is 0.017 min^{-1} and the half-time is 41 min.

Analogous studies were done with *E. coli* Ile- and Val-tRNA synthetases at 0 °C. For Ile-tRNA synthetase, it was shown that, from 0 to 80 mM bromouridine, the rate constant is linearly dependent on concentration (data not shown), as expected for a pseudo-first order process. The rate constants for inactivation for the two enzymes are within $\pm 10\%$. This quantitative agreement further establishes the universality of the uridine site on synthetases.

No inactivation occurs with bromouracil, 5-bromo-2'-deoxyuridine or with 5'-bromouridine-5'-monophosphate. Specificity for uracil ribosides has been noted previously in the synthetase-catalysed deribosylation reaction¹¹.

Stoichiometric binding of nucleoside to synthetase accompanies inactivation

Ala-tRNA synthetase was incubated with 50–80 mM [^{14}C]bromouridine for times sufficient to achieve >90% inactivation. The resulting mixture was then applied to a Biogel P-30 column equilibrated with 60 mM unlabelled bromouridine and was eluted with the same buffer. The unlabelled halopyrimidine effectively prevents artefacts arising from nonspecific, non-covalent association of pyrimidine with synthetase.

Figure 3 shows the results. Biogel P-30 provides excellent resolution of nm-enzyme-bound nucleoside, and association of nucleoside with enzyme is obvious. For the experiment shown in Fig. 3 0.86 mol of nucleoside is bound per catalytic site of synthetase.

Table 2 Reactivation of 5-bromouridine inactivated Ala-tRNA synthetase

Time (min)	Fractional activity	
	(+) DTT	(-) DTT
0	0.33	0.33
30	0.72	0.30
60	0.92	0.40

Incubation and reaction conditions for aminoacylation were as described in Fig. 2a legend. After 65 min incubation (time 0) the synthetase-bromouridine mixture was diluted 40-fold and incubated with or without 10 mM DTT at 24 °C. All values are averages of two determinations.

Further data on the association of bromouridine with synthetase are given in Table 1. Uridine, which in contrast to bromouridine does not inactivate after prolonged incubation with enzyme (data not shown), does not bind. The sulphhydryl reducing agent DTT, which prevents inactivation (see below), also prevents binding. Finally, incubation with bromouridine for a time which gives 50% inactivation in turn yields 0.59 mol of nucleoside bound per mol of catalytic site. Thus, inactivation of synthetase correlates well with binding of the pyrimidine nucleoside.

The time dependence of inactivation and of binding, and the failure of excess unlabelled bromouridine to displace bound radioactive nucleoside, suggest that nucleoside association with enzyme is covalent. Consistent with this conclusion is the failure of SDS treatment of the enzyme complex to displace nucleoside from the enzyme (Table 1).

A sulphhydryl group such as cysteine SH is a candidate for nucleophilic attack at pyrimidine C-6 and Michael adduct formation^{15–17,21,25,26,29,32–38}. However, phosphorylase—a cysteine-rich protein of molecular weight similar to that of Ala-

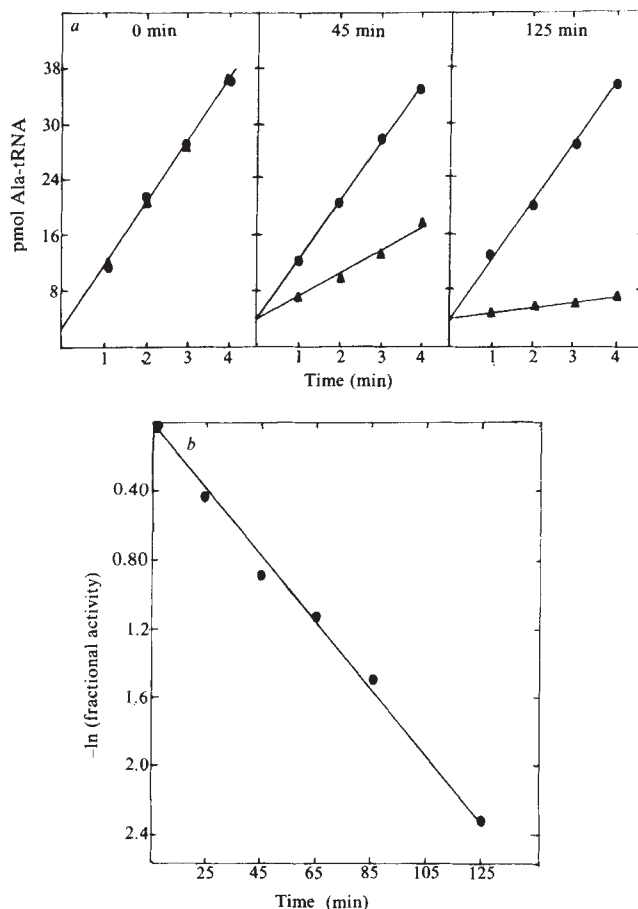


Fig. 2 a, 5-Bromouridine-mediated inhibition of AlaRS aminoacylation activity. *E. coli* Ala-tRNA synthetase was purified following the procedure of Putney *et al.*⁴⁸ and was estimated as $\geq 70\%$ pure on SDS-gel electrophoresis. ^3H -alanine (30 Ci mmol^{-1}) was from ICN. Reaction mixtures ($100 \mu\text{l}$) contained 40 mM NaPO_4 buffer pH 7.5, 3 mM ATP, 10 mM MgCl_2 , 20 mM KCl, 0.1 mg ml^{-1} bovine serum albumin, 2 mg ml^{-1} crude tRNA from *E. coli* B (Schwarz-Mann), 15 μM amino acid and 5 μM radiolabelled amino acid. Activity was determined by measuring the incorporation of labelled amino acid into trichloroacetic acid (5%) precipitable counts as a function of time⁵². Filter pads (Whatman 3 MM) were placed in 5 ml of toluene-based scintillation fluid and counted for 1 min in a Beckman LS 100-C liquid scintillation counter. Time points were taken at 1, 2, 3 and 4 min. The precipitable counts represented 20% of the total possible. For inactivation, incubation mixtures contained 80 mM 5-bromouridine (Sigma), 10 mM MgCl_2 , 20 mM NaPO_4 pH 7.5, and 1–2 μM synthetase. DTT was removed from synthetases by passage over a Biogel P-30 column in 20 mM NaPO_4 at 4 °C. The final non-inhibitory concentration of 5-bromouridine in the assay mixture was 5 mM. Incubations and assays were done at 24 °C. $\circ$, Control; Δ , 5-bromouridine incubation. b, Kinetics of inactivation of Ala-tRNA synthetase by 5-bromouridine. Data are averages of three determinations. See legend to a for details.

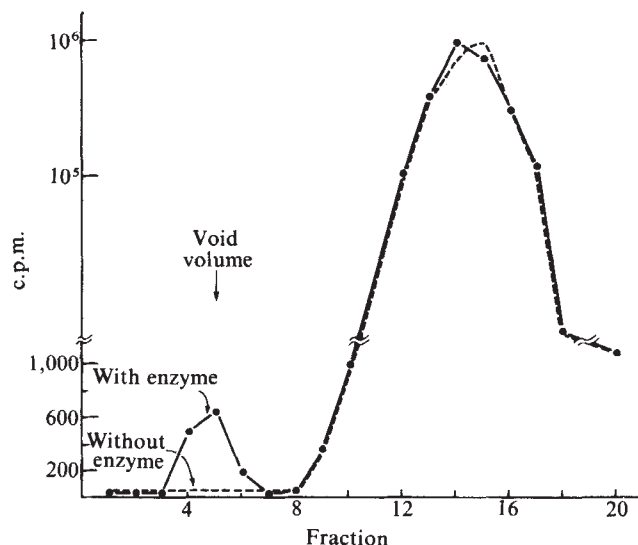


Fig. 3 Chromatography of an AlaRS-nucleoside complex. ^{14}C -5-bromouridine was synthesized from $[\text{U-}^{14}\text{C}]$ uridine (Amersham 450-540 mCi mmol^{-1}). The procedure was modified from Visser *et al.*⁵³. The reaction mixture contained 20 $\mu\text{Ci } ^{14}\text{C}$ -uridine, 2% ethanol and 40 μl bromine in 1 ml total volume. The reaction proceeded for 5 h with constant stirring at room temperature in the dark. After lyophilization, the material was purified in the dark by either two- or one-dimensional TLC on Brinkman cellulose plates (0.1 mm) using *n*-butanol: H_2O , 86:14 (ref. 34). The radioactive spots were scraped and eluted with $2 \times 2 \text{ ml H}_2\text{O}$ at room temperature. The isolated material chromatographs with authentic 5-bromouridine in two solvent systems³⁴. The yield is $\sim 10\%$ with purity ranging over 75–100%. The major impurity is a species which does not move from the origin. Uracil is not a detectable contaminant. To arrest degradation, the material was stored in 10% ethanol at -70°C , and always used within 3 days of synthesis. Incubations were at 24°C , 20 μM Ala-tRNA synthetase, 10 mM MgCl_2 , 20 mM NaPO_4 pH 7.5 and 50–80 mM 5-bromouridine. Enzyme concentration was determined by ATP-PP_i exchange activity (see Fig. 5 legend and ref. 48). Incubation times were long enough to inactivate $>90\%$ of the synthetase. The mixture ($\sim 220 \mu\text{l}$) was applied to a Biogel P-30 column (3 ml) equilibrated with 20 mM NaPO_4 and 60 mM bromouridine at room temperature. Fraction size was small enough to effect a clean separation between radioactivity appearing in the void volume and that in the included volume. The void volume was determined by chromatography of a myoglobin solution (10 mg ml^{-1}). Fractions were counted in 5 ml Aquasol (NEN) on a Beckman LS 100-C scintillation counter. As recovery of enzymatic activity from Biogel P-30 is $>95\%$, no correction was made for retention on the column.

tRNA synthetase⁴⁰—does not stably associate with bromouridine (Table 1). This confirms earlier work which showed that stable covalent protein-pyrimidine interactions are not easy to generate as artefacts^{9,11}. Note also that the reaction between synthetases and 5-bromouridine is far more rapid than that observed in model systems of 5-halopyrimidines and sulphydryl compounds³⁴.

Release of uridine from enzyme regenerates activity

As stated above, the presence of sulphydryl reducing agents (such as 10 mM DTT) blocks the inactivation of synthetase caused by prolonged incubation with 50–80 mM bromouridine. This suggests that covalently labelled, inactive enzyme might be reactivated on incubation with a sulphydryl reagent. The data in Table 2 show this to be true. Ala-tRNA synthetase was incubated with 80 mM bromouridine until 33% activity remained. Excess bromouridine was diluted out and aminoacylation activity was assayed after incubation of the enzyme for various times in the presence and absence of 10 mM DTT. In the absence of sulphydryl reagent there is little change in activity for 60 min, but in its presence activity returns in a time-dependent way.

Because inactivation accompanies covalent binding of nucleoside, the release of nucleoside is expected to occur on reactivation. A stoichiometric enzyme-radiolabelled nucleoside complex was isolated on Biogel P-30 as described above (see

Fig. 3 legend). The complex was incubated with 10 mM DTT for 1 h, the protein ethanol precipitated and the radioactive supernatant analysed by chromatography on cellulose thin layer plates. (If DTT is not added, all radioactivity precipitates with protein. Stability to ethanol precipitation is further evidence that the enzyme-nucleoside adduct is covalent.) The results of chromatography are shown in Fig. 4. The chromatographic system separates bromouridine and uracil from uridine and bromouracil, the predominant product being uridine. A small amount of bromouridine is also obtained and control experiments show that this is at the level of 'background' found when the enzyme is incubated with bromouridine for times too short to achieve inactivation. For the experiment shown, 70% of the total radioactivity originally bound to the enzyme is recovered as uridine; the remainder either appears elsewhere on the chromatogram or is lost during work-up of the chromatographic system.

These results further confirm that inactivation is associated with covalent linkage of pyrimidine nucleoside to enzyme. As stated earlier, a probable mechanism is rearrangement of the initial Michael adduct by attack of a secondary enzyme nucleophile at C-5 to release bromide. Treatment of the resulting adduct with reduced sulphydryl gives enzyme and free uridine³⁴. The attacking nucleophiles may be enzyme sulphydryl groups. (Earlier work has demonstrated the importance of sulphydryl groups for synthetase activity^{41–46}. A cysteine residue has also been implicated as the attacking nucleophile in the interaction of thymidylate synthetase with 5-fluoro-2'-deoxyuridylate^{21,23,25,26}.) Other schemes for uridine production from bromouridine after Michael addition and treatment with sulphydryl reagents are possible and cannot be rigorously excluded^{32–38}. These details of mechanism, while of interest, are not essential for the main conclusions established here.

Polypeptide segment required for the tRNA-dependent step is necessary for uridine attachment

Aminoacylation of tRNA occurs in two steps: condensation of amino acid with ATP to give enzyme-bound aminoacyl adenylate, and reaction of bound adenylate with cognate tRNA to give aminoacyl tRNA^{1–4}. Inhibition of either step will block aminoacylation of tRNA; specific inhibition of the second step could allow aminoacyl adenylate formation to proceed without subsequent reaction with tRNA.

Covalent interaction of nucleoside with Ala-tRNA synthetase blocks the aminoacylation of tRNA (Fig. 2a,b). Bromouridine inhibition of adenylate formation, which can be

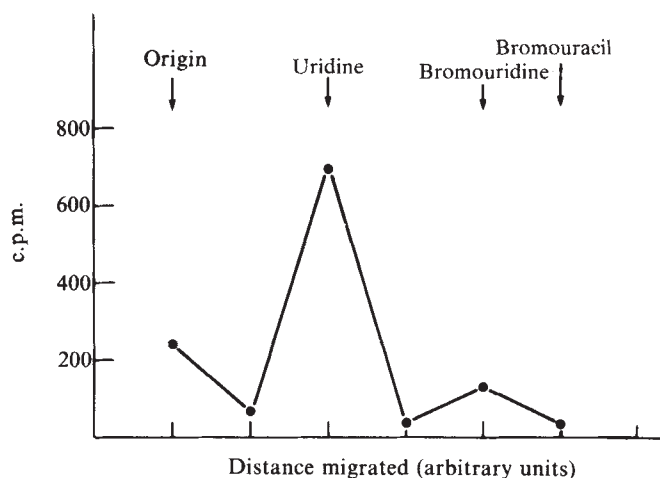


Fig. 4 Release of uridine from the inactive enzyme-nucleoside complex. Inactivation and column chromatography were performed as described in Fig. 3 legend except that the column was equilibrated only with 1 mM NaPO_4 , pH 7.3. DTT to 10 mM was added to the enzyme-containing fractions for 1 h at 24°C . After TLC (Fig. 3), spots were scraped and counted in Aquasol. The rest of the protocol is as described in the text.

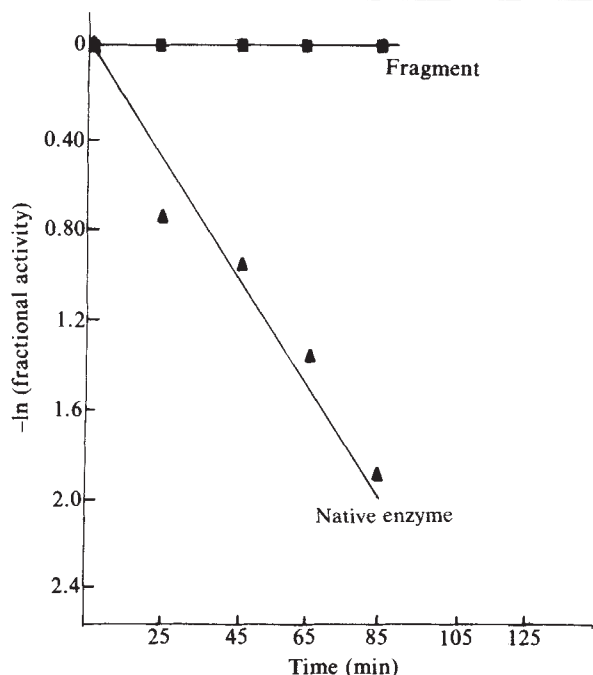


Fig. 5 Inactivation of native Ala-tRNA synthetase and fragment. Details for inactivation are described in Fig. 2a legend. Ala-tRNA synthetase fragment was prepared as described in Putney *et al.*⁴⁸. For PP_i-ATP exchange (assay adapted from refs 48, 54), 750- μ l reaction mixtures contained 25 mM Tris-HCl pH 7.5, 2 mM ATP, 2 mM amino acid, 2 mM NaPP_i (10 mCi mmol⁻¹, NEN), 10 mM KF, 5 mM MgCl₂, 10 mM mercaptoethanol. Time points were taken at 15, 19, 23 and 27 min. All experiments were done at 24°C. Data represent the average of three determinations.

monitored independently by the ATP-PP_i exchange assay, parallels that of aminoacylation (see below). The rate constants for inactivation are similar. Therefore, inactivation of aminoacylation can be explained by inhibition of adenylate synthesis, and not necessarily by blockage of a step which requires tRNA, as might be expected.

To explore this issue further, advantage was taken of structural information available on Ala-tRNA synthetase. The entire 875-amino acid sequence has been determined by a combination of DNA sequencing of the gene for the enzyme and amino acid sequencing of the protein itself⁴⁷⁻⁴⁹. These and related studies showed that full activity for aminoacyl adenylate synthesis is contained within the 440-residue N-terminal half which can be isolated as a stable fragment⁴⁷⁻⁴⁹. This fragment, while having the same ATP-PP_i exchange activity as the native polypeptide, does not catalyse aminoacylation of tRNA⁴⁸. This suggests that the C-terminal half is required for the tRNA-dependent step. Recent segment-directed mutagenesis studies suggest that a relatively short stretch of 30-60 residues in the C-terminal half is needed (M. Jasin and P.R.S., unpublished). If uridine interacts with the N-terminal half, reaction of the 440-residue N-terminal fragment with bromouridine should inactivate the synthetase. Figure 5 shows the results of incubation of native Ala-tRNA synthetase and the aforementioned fragment for varying times with bromouridine. In contrast to Fig. 2, these data deal with inhibition of ATP-PP_i exchange. As stated above, adenylate synthesis by native enzyme is inactivated by prolonged exposure to bromouridine. The N-terminal fragment is not inactivated, however, even after exposure for times which give full inactivation of the native enzyme. This implies that the uridine interaction site is not in the polypeptide portion required for adenylate synthesis, but rather is in the C-terminal part required for the tRNA-dependent step.

Further confirmation was obtained from investigation of the deribosylation reaction. As described earlier, reaction of synthetases with bromouridine gives a Michael adduct which frequently decomposes to produce bromouracil and ribose¹¹. We found that this reaction occurs with native Ala-tRNA synthetase, but not with the fragment (data not shown).

Inhibition of adenylate synthesis by covalent attachment of nucleoside to native enzyme presumably occurs because of communication between the N- and C-terminal halves of the protein. If tRNA binds to a C-terminal portion while adenylate synthesis is on the N-terminal half, interaction between segments must occur in order to achieve aminoacylation. Isolated N-terminal fragment is free from constraints imposed by the part of the polypeptide required for the tRNA-dependent step, and therefore is not inactivated by exposure to bromouridine. Further evidence for communication between these separate regions of the protein comes from studies of substrate protection from 5-bromouridine-induced inactivation. For example, alanine or ATP alone provide partial protection and together give nearly full protection⁵⁰.

Concluding remarks

The main conclusion established here is that the site on aminoacyl tRNA synthetases which interacts with the uracil ring—a site identified on all synthetases tested to date⁹⁻¹¹—is essential for catalytic activity. A second conclusion is that, for Ala-tRNA synthetase, this site is located on the segment of polypeptide required for reaction with tRNA, and not the segment associated with aminoacyl adenylate formation. Therefore, the results are consistent with the hypothesis that this site interacts with tRNA⁹. Because aminoacyl tRNA synthetases catalyse H-5 exchange at the common uridine 8 (refs 9, 10), this is presumably the site of interaction. Final confirmation awaits isolation of a stable covalent enzyme-tRNA complex.

A role for covalent protein-nucleic acid interaction in the recognition and aminoacylation of tRNA has been discussed previously^{2,9,10}. All known modifications of uridine-8 (such as the U-8-C-13 cross-link) do not block the site on uracil (C-6) which could interact with the enzyme^{2,9,51}. Preliminary results suggest that specific excision of the base from the 8 position results in inactivation of tRNA (H. J. P. Schoemaker, C. Bedrosian and P.R.S., unpublished), although this inactivation could be for reasons other than a requirement for the synthetase to bind covalently to this base (for example, an inactivating conformational change in the tRNA).

Michael addition of a protein nucleophile to the uracil ring is a key feature of the mechanism of thymidylate synthetase^{12,13,20,30}. It is almost certainly part of the mechanism of dCMP hydroxymethylase¹⁴ and dUMP hydroxymethylase²⁰. At the nucleic acid level, pseudouridylate synthetase³⁰, uracil-N-glycosidase³⁰, ribothymidylate synthetase and DNA methylases all catalyse reactions which can be neatly accommodated by Michael addition schemes. Aminoacyl tRNA synthetases may in a primitive way be connected with these other systems. In general, future work may show protein-pyrimidine Michael adducts to be components of other protein-nucleic acid complexes.

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LETTERS TO NATURE

Extended radio structure of BL Lac type objects

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BL Lac type objects^{1,2} have many properties in common with highly polarized quasars, and indeed appear to be distinguishable from these more active of the quasars only by the absence of prominent optical emission lines and perhaps the extremeness of their variability^{3,4}. It has been suggested that BL Lacs and active quasars should be regarded as one family of active objects, termed Blazars³, in which special geometrical effects and probably relativistic beaming^{5,6} have an important role. Crucial to such suggestions is the radio structure of the sources^{4,5}, and we report here observations which show that the BL Lacs do indeed possess the general core-halo morphology predicted by the Blazar models, although there is an important difference to the quasars in that compact jet-like features are much less conspicuous.

The radio structures of both active quasars⁷ and BL Lac type objects⁸ are dominated by compact nuclear cores, and it is these which are responsible for the characteristic centimetre excesses in the radio spectra of the sources. Jets and diffuse haloes have recently been detected around the cores of the quasars⁹ but although the BL Lac type objects are often known to be extended on the scale of a few arc seconds^{8,10}, in only a few sources has the detailed radio morphology been established. To rectify this we have investigated the arc-second radio structure of a sample of 11 BL Lac type objects from the catalogue of Hewitt and Burbidge¹¹.

The observations were made between 1978 and 1980 and all sources were tracked for long periods with the 127-km Jodrell to Defford radio-linked interferometer at 0.41 GHz. More limited observations were made with the same instrument at 1.67 GHz and many sources were also observed with the shorter spacing Jodrell to Wardle interferometer at 0.96 and 1.67 GHz. The visibility data were assembled on the *u-v* plane and analysed in the manner described by Stannard *et al.*¹² to give the structural information summarized in Table 1. Where there are previous interferometric observations^{8,10,13–19} our data are compatible, and we have combined these generally higher frequency measurements with our own to derive spectral indices for the various structural features over the frequency range 0.4 to ~5 GHz.

In all but two of the objects studied there are compact nuclear cores with flat or inverted radio spectra. The exceptions are 2207+020, which has a relatively compact but apparently normal spectrum core, and 2335+031, where we can only place an upper limit of 0.20 to the fraction of the 0.4 GHz flux in any sub arc-second structure. There is, however, a VLBI detection of 2335+031 at 5 GHz (ref. 20), which if attributable to a flat spectrum core would give a visibility of 0.03 at 0.4 GHz.

Seven of the 11 objects are found to possess extended structure with a spectral index of about -0.8, and it is this which is responsible for the overall steep spectrum of many of the sources and for the much lower fractions of flux contained in the cores at 0.4 GHz compared with higher frequencies^{8,10}. The presence of extended emission does not seem to be a function of either source redshift or luminosity as indicated by the redshift estimates¹¹ available for eight of the objects in our sample. What stands out very clearly from our observations, however, is that when extended emission is present it is diffuse, with a visibility on the *u-v* plane which just trails away with increasing projected spacing and which, with the exception of 1400+162, shows no evidence of any oscillation which would indicate the presence of compact structure away from the nuclear core. Typically the extended emission has an ellipticity of about 3:1, and in most cases it appears nearly concentric with the core although there are examples of more one-sided emission which could perhaps be described as a relaxed jet.

Subsequent to the observations with single baseline interferometers, three of the objects with particularly weak or unusual radio structure were re-observed with the multi-element radio-linked interferometer network²¹ (Merlin) at Jodrell Bank at a frequency of 0.4 GHz and the results are displayed in Fig. 1. Only the outermost four elements of the array were used to map 1400+162 and so there is insufficient spatial frequency coverage to reveal the diffuse extended emission which stretches to about 12 arc s. on each side of the core^{16,17}. The observations reveal clearly, however, (Fig. 1a) that the compact asymmetric structure close to the nucleus evident in the single baseline observations is in fact a curved jet extending to the east of the core. We can also place an upper limit of <1% to the fraction of the source flux contained in compact hot spots further out in the structure, and so even though the extended emission has a vaguely double morphology, it may perhaps be better described as a diffuse halo. The small-scale extended emission surrounding the cores of both 0235+164 and AP Lib is confirmed in the Merlin observations (Fig. 1b, c), although in both sources the amount of halo emission is appreciably less than previously reported^{18,19}.

The core-halo morphology of the BL Lac type objects appears very different from the collimated twin outer lobe

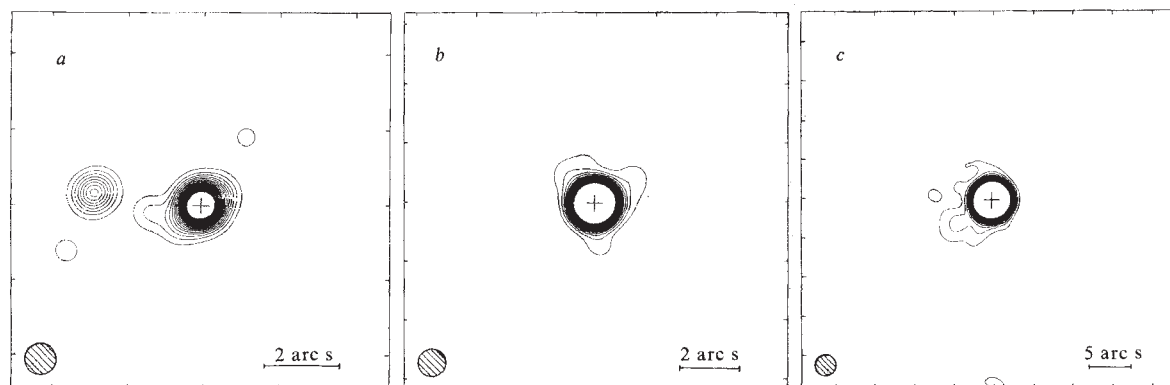


Fig. 1 Merlin maps of the BL Lac objects 1400+162 (a), 0235+164 (b) and AP Lib (c) at 0.4 GHz. The contours are $(1, 2, 3, \dots, 20) \times N\%$ of the peak brightness B_0 in mJy per synthesized beam, with $N=3$ and $B_0=253$ for 1400+162, $N=1$ and $B_0=1,070$ for 0235+164 and $N=0.5$ and $B_0=2,200$ for AP Lib. On each map north is to the top and east to the left, and the half power widths of the synthesized beams of 0.9, 0.9 and 2.5 arc s. respectively are represented by the shaded circles. Our phase stable single baseline observations indicate that the centres of the compact cores (denoted by crosses) coincide with the optical nuclei.

structure commonly found in radio galaxies and steep spectrum quasars. Although a few of the BL Lacs have a luminosity below the Fanaroff and Riley²² borderline, most are of such a luminosity that any extended structure would be expected to exhibit prominent outer hot spots²³. The absence of such features in the BL Lacs might therefore be construed as an indication of some form of disruption of the supply beams²⁴ to the extended lobes, perhaps caused by a particularly rich inter-cluster environment (and indeed some objects such as 3C66A²⁵ and 1400+162¹⁵ are known to be situated in small groups of galaxies) or reflecting collimation or stability problems attributable to the lack of turbulent plasma in the nuclear regions^{2,24}. There is, however, still a problem in explaining why the core radio emission of the BL Lacs is typically several orders of magnitude stronger than that from the nuclei of classical double sources, and indeed approaches the luminosity of the cores of the active polarized quasars.

The radio properties of the cores are similar in the BL Lacs and active quasars, but what about the overall morphology of the objects? We investigate this in Table 2 by comparing our results on the BL Lacs with the corresponding structural detail in the quasars in the sample of core dominated sources recently studied⁹ using Merlin at 0.4 GHz. The cores are the most prominent features in both types of object, and within the

limitation of small number statistics both types possess similar fractions of diffuse extended emission. There is, however, a major difference between the two in that any compact one-sided 'jet' emission is relatively much weaker in the BL Lacs, if it is present at all. We emphasize that this result is not an artefact of the differing analysis techniques as the core and asymmetric jet morphology of the quasars is readily apparent in observations²⁶ of comparable quality to our own.

What then has happened to the compact jet structure in the BL Lac type objects? Jet morphology is known to depend on source luminosity²⁷, but whilst the luminosity of the BL Lacs is on average probably only about a third that of the quasars as a consequence of their lower redshifts¹¹, there is no suggestion of a luminosity range among radio galaxies and quasars in which jets are absent altogether. We thus look to something special about the BL Lacs themselves, and have already mentioned possible differences in their environment which may affect the collimation of material flowing out from the nuclear regions. In the specific comparison with quasars it should also be noted that there is the possibility of a difference in the nature of the underlying galaxy^{2,28} and that this may fundamentally affect the emission may be evidenced by the marked contrast in the ratio of the core to galaxy luminosity of the objects at optical wavelengths²⁸.

Table 1 The radio structures of the BL Lacs

Source	0.4 GHz flux density at epoch 1979.1 (Jy)	Core			Ref. to published arc s structure measurement	Extended structure		
		Fraction of flux at 0.4 GHz	Angular size (arc s)	Spectral index ($S \propto f^\alpha$)		Angular size (arc s)	Morphology	Spectral index
0219+428 3C66A	5.0	0.08	<0.1	+0.2	8, 12	35×12 in PA 135°	Diffuse concentric halo	-0.8
0235+164	1.2	0.95	<0.3	+0.0	8, 18	~2	Concentric halo	
0422+004	0.7	1.00	<0.1	+0.0	8			
0846+513	0.2	1.00	<0.3	+0.0				
0851+202 OJ287	1.3	1.00	<0.3	+0.4	8			
1101+384 MKN421	1.2	0.53	<0.3	+0.0	8, 13, 15	210×60 in PA 115°	Diffuse concentric halo	-0.7
1400+162	2.0	0.12	<0.1	+0.1	8, 16, 17	25×15 in PA ~110°	Diffuse lobes + inner jet	-0.8
1514-241 AP Lib	2.8	0.93	<0.3	+0.2	8, 19	7×2 in PA 127°	Diffuse jet?	(-0.7)
2200+420 BL Lac	2.6	1.00	<0.1	+0.3	8			
2207+020	2.1	0.45	0.2	-0.8	14	~4	Diffuse concentric halo	-0.9
2335+031	3.2	<0.2	<0.1		8, 18	2×1 in PA 144°	Diffuse concentric halo	-0.7

Table 2 Comparison of the radio structures of BL Lacs and quasars

	Fraction of 0.4 GHz flux contained in:		
	Core	Compact jet	Diffuse halo
Core dominated quasars	0.49	0.31	0.20
BL Lac type objects	0.65	≤0.05	0.30

On the Blazar scheme³⁻⁵ the BL Lacs and highly polarized quasars are objects where a central active core is viewed directly down the 'throat' of material channelling out of the nuclear regions. The prominence of the radio cores is then attributed to enhancement of the emission due to relativistic bulk motion, and the jets and extended emission presumably occur where the beams diffuse or interact with the surrounding medium. Differences between the BL Lacs and quasars are attributed to the quasars lying more oblique to the line of sight, and since

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curvature of the jets is known to occur^{29,30} it may be that a combination of geometrical and beaming effects could result in the jets being less noticeable in the BL Lacs. It is, however, difficult to construct simple models which either merge the cores and jets in the BL Lacs without steepening the nuclear spectrum or which reduce the emission from spatially separate jets by the necessary amount. An attraction of the scheme (and also its extension to a unified source theory⁹) is, however, an explanation for the diffuse emission surrounding the cores of BL Lacs and quasars, and in this context we note that the properties of the halo emission in the BL Lacs (a spectral index of ~ -0.8 , a linear size of between 20 and 300 kpc and an integrated radio luminosity between 10 MHz and 100 GHz in the range 10^{41} – 10^{44} erg s⁻¹) are quite consistent with the outer lobe structure of a classical double source being viewed 'end on'. Despite the problem of explaining the absence of jets we thus take our observations of the extended structure of BL Lac type objects as support for the Blazar hypothesis.

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Volcanism and igneous processes in small icy satellites

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The small saturnian satellites exhibit a remarkable diversity of surficial features¹⁻³ including many which appear to be a consequence of internal activity: 'wispy' terrain, linear troughs, and terrains displaying large gradations in crater density. I argue here that the most likely cause of endogenic processes in these bodies is the igneous activity associated with a low melting point NH_3 - H_2O magma, a possibility first pointed out by Lewis⁴. Radiogenic heating probably suffices to provide the necessary melting in Tethys, Dione, Rhea and Iapetus whereas tidal heating is needed for Enceladus and possibly Mimas. Migration of large fluid-filled cracks is likely (as in Weertman's theory for water-filled crevasses in glaciers⁵), leading to surface flooding. If clathrates are present, as first suggested by Miller⁶, then pyroclast-forming explosive events can occur at a near-surface intrusive contact between magma and one or more of the methane, nitrogen and argon clathrates.

The environment in which these satellites formed was partly determined by Saturn and would have had a much higher gas pressure than the solar nebula, perhaps of the order of 1 bar. The sequence of important icy condensates in an equilibrium condensation⁷ is then H_2O (~ 240 K), NH_3 - H_2O (~ 130 K), CH_4 - H_2O (~ 90 K), Ar - H_2O (~ 70 K) and also N_2 - H_2O (~ 90 K) if molecular nitrogen is present, as seems possible⁷. A common supposition is that the temperature profile in a gaseous disk emplaced around Saturn would preclude the incorporation of ices more volatile than water ice except in the more distant satellites (including Titan) but unlike the galilean satellites, there is no clear evidence for a radial gradient of uncon-

pressed densities in the saturnian satellites². Furthermore, buffering by the background radiation bath of the solar nebula or newly formed Sun can provide an isothermal disk, if the intrinsic luminosity of the disk is low⁸. It is also possible that the compact group of satellites inside Titan's orbit formed in a cooler, more confined disk, substantially later than the formation of Titan (which is $>95\%$ of the mass in the satellite system). This suggestion is prompted by the large gap between Titan and the inner satellite system, and the suggested collisional evolution and reaccumulation of the small satellites². In the absence of relevant observational data, there are no strong arguments either for or against the presence of ammonia and perhaps even more volatile ices (clathrates) in all or most of these satellites. The models described here assume incorporation of at least NH_3 .

These satellites probably formed well after the decay of ^{26}Al and are too small for accretional heating to be important. A likely initial state is a homogeneous mixture of particulate silicates ($\sim 40\%$ of the mass) and ices ($\sim 45\%$ of the total mass as H_2O , $\sim 15\%$ NH_3 as a hydrate; all or part of the water ice fraction could include CH_4 , N_2 and Ar as clathrates). Consolmagno and Lewis⁹ modelled the evolution of such bodies with conservative assumptions which minimize the likelihood of melting; I have performed similar calculations assuming only

Table 1 Thermal characteristics of the models

	Class I	Class II*
Thermal conductivity ($\text{W m}^{-1} \text{ deg}^{-1}$)	380/T	190/T ($0 < x < 0.995$) 1.9×10^{-3} ($0.995 < x < 1$)
Specific heat ($\text{J kg}^{-1} \text{ deg}^{-1}$)	8.5T	8.5T

(T is temperature in K, x is fractional radius)

* The values of regolith conductivity and regolith thickness can be varied without significantly changing the results, provided their ratio remains constant.

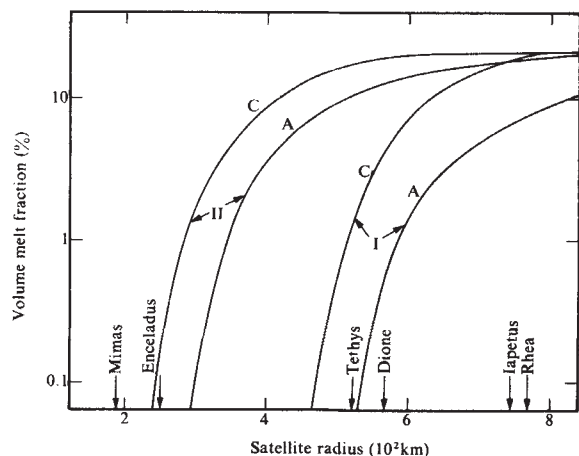


Fig. 1 Volume percentage of eutectic melt produced as a function of satellite radius. Case I is conservative (high conductivity, no regolith) and case II is possibly more realistic (lower conductivity, regolith present). C, the centre of the satellite; A, the average volume melted for the whole satellite.

conductive transport, but allowing for the likelihood of a lower thermal conductivity than they assumed and the possibility of a very low conductivity regolith. Two classes of models were considered and their characteristics are given in Table 1. Class I is conservative in assuming the conductivity and heat capacity of a mixture of crystalline phases, and neglecting the low conductivity clathrates. Class II is an arbitrary but plausible modification which allows for the reduction in conductivity caused by the presence of dust particles, defects, vitreous or amorphous phases^{10,11} and clathrates¹². Class II also includes a thick non-annealed impact-generated regolith (likely for the low temperatures and pressures of these bodies). The thermal conductivity and specific heat of water ice were taken from ref. 13. The thermal conductivity of $\text{NH}_3\cdot\text{H}_2\text{O}$ does not appear to have been measured but published specific heat¹⁴ and IR spectral¹⁵ data indicate a substantially lower effective Debye temperature than water ice and an application of Callaway's model¹⁶ suggests a lower thermal conductivity by about a factor of two at the temperatures of interest. The thermal conductivity of the mixture was estimated from a geometric mean¹⁷. A finite difference method was used to solve the heat conduction equation, assuming an initially isothermal state at $T = 80\text{ K}$ (the assumed surface temperature). Radiogenic heating appropriate for C1 carbonaceous chondritic material (as tabulated by Stacey¹⁸) was assumed for the disseminated silicates.

Because the main point of these calculations is to establish the extent to which a eutectic melt ($T \sim 175\text{ K}$, 66% H_2O –34% NH_3) can form, subsolidus convection is not likely to be important because of the high viscosity of water ice at 175 K. Although substitution of NH_3 in the water ice lattice may slightly soften the ice¹⁹ and although $\text{NH}_3\cdot\text{H}_2\text{O}$ may have a lower viscosity, the rheology of the mixture is likely to be dominated by water ice. Partial melting will always occur at 175 K for any mole fraction of NH_3 less than the water abundance (cosmic abundance satisfies this constraint). Melting of pure water ice would never occur because of the buffering by subsolidus convection²⁰. Excess methane (not bound as a clathrate) is not considered because of the extremely low temperature ($\sim 40\text{ K}$) required to condense it and because it would escape very early (the first 10^8 yr after satellite formation) and any evidence of its volcanism would have been obliterated by subsequent impacts.

The results of the calculations are summarized in Fig. 1. The volume percentage of melt is determined by assuming that all the heating in excess of 175 K goes into overcoming the latent heat of the eutectic mix, about 32 cal g^{-1} (ref. 21). The real situation is probably intermediate between the two classes of models considered and allows significant melting by radiogenic heating in all of the satellites except probably Mimas and

Enceladus. Figure 2 shows the radial extent and timing of the partial melting for two satellite radii: 540 km (representative of Tethys and Dione) and 740 km (representative of Rhea and Iapetus). The timing of the initiation and peak of partial melting activity is compatible with the inferred resurfacing episodes at around the time of terminal bombardment, especially for models possessing a regolith. These models do not include melt migration (see below) and therefore provide only an approximate guide to the timing of volcanic activity.

Evidence for endogenic activity in Mimas is slight. Some partial melting is possible by tidal heating if the eccentricity of Mimas was rapidly damped from ~ 0.1 to its present value of 0.0201 in the first few hundred million years. Application of the analysis of Peale *et al.*²² indicates that this would lead to the release of about $10^{10}\text{ erg cm}^{-3}$, equivalent to a temperature rise of several hundred degrees. Rapid damping (which requires a primordial tidal Q much less than the present value $\sim 10^3$) is needed to ensure retention of a large fraction of this heat since the conductive cooling time of Mimas is short.

Enceladus has striking evidence for geologically recent endogenic activity, clearly requiring tidal heating. I shall not consider Enceladus further here, as it requires careful consideration of the orbital evolution and the tidal Q s of both the satellite and the planet²³, except to remark that $\text{NH}_3\text{--H}_2\text{O}$ activity has a substantially lower energy cost than the melting of pure water ice, an important point in view of the meagre tidal energy budget for Enceladus.

The viscosity of the $\text{NH}_3\text{--H}_2\text{O}$ melt is much higher than that of liquid water²⁴, possibly $\sim 10^2\text{ P}$. The melt is less dense than the coexisting solid and will migrate upwards along channels between ice grains. This problem is analogous to the differentiation of eucrite parent bodies²⁵. Application of D'Arcy's law suggests melt velocities of $10^{-3}\text{--}10^{-5}\text{ f}^2\text{ cm s}^{-1}$, where f is the melt fraction, and the flow is rapid on the relevant time scale (10^8 yr) provided f exceeds a few per cent. The melt cannot percolate higher than the 175 K isotherm, but can accumulate to form an asthenospheric layer or magma chambers. Diapirism (that is, Rayleigh–Taylor instabilities) is found to be ineffective because of the high viscosity ($10^{22}\text{--}10^{24}\text{ P}$) of the neighbouring ice, but crack nucleation and growth is possible because of the tensional environment provided by thermal expansion. The theory of fluid-filled buoyancy-driven cracks^{5,26} predicts that the maximum stable vertical length of a crack is $2[K_{IC}/g\Delta\rho\pi^{1/2}]^{2/3} \sim 10\text{--}100\text{ km}$ if the stress intensity factor $K_{IC} \sim 10^{10}\text{ erg cm}^{-5/2}$, comparable with that of rock (g is the gravitational acceleration, and $\Delta\rho$ is the ice–magma density difference). The corresponding crack width is $10^2\text{--}10^3\text{ cm}$. Note that these cracks are larger on small (low g) bodies: igneous

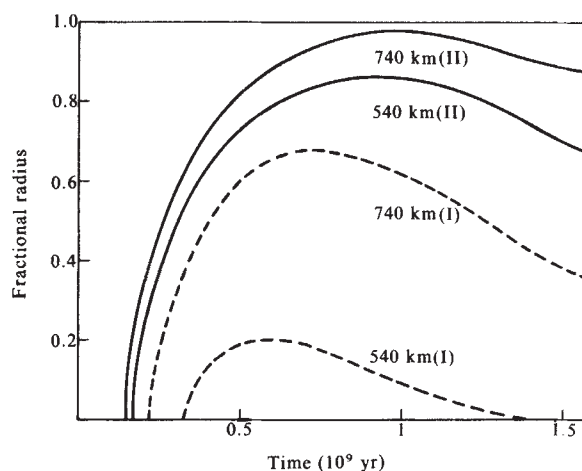


Fig. 2 Fractional radius of the satellite out to which the partial melting occurs (assuming no melt migration) as a function of the time since satellite formation. The calculations are for two satellite radii (540 km, 740 km) and the two classes of models (Table 1). Terminal bombardment for the Moon occurred at around $7 \times 10^8\text{ yr}$; a similar timing might apply to these satellites^{2,3}.

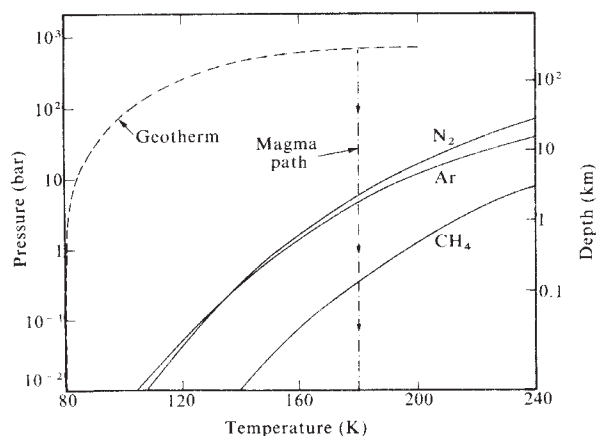


Fig. 3 Solid lines denote the pressure below which the respective clathrates decompose into water ice and gas. The dashed line is a typical temperature profile for a satellite interior (surface temperature, 80 K). The dash-dotted line is a path of $\text{NH}_3\text{-H}_2\text{O}$ magma rising along an opening crack. The depth scale corresponds to Iapetus (central pressure ~ 900 bar).

events were thus large but infrequent compared with terrestrial planets. A previous attempt to understand the velocity of fluid-filled cracks failed²⁷, but a new theory²⁸ predicts a velocity of $1\text{--}10\text{ cm s}^{-1}$ and the crack can open all the way to the surface with $\approx 10\%$ freezing of the fluid. The resulting surface lava flows would be analogous to basaltic flooding on terrestrial bodies (the viscosity is lower, but this is compensated by the lower gravity). There may be intrusive events as well as extrusive; the total amount of magma available (as given by Fig. 1) could provide ~ 10 km surface layer if extrusive events dominated. Rapid quenching of lava would lead to metastable $\text{NH}_3\text{-H}_2\text{O}$ glass¹¹ which has very low thermal conductivity, thereby thermally insulating the interior and promoting further melting at depth. Glass formation is likely as the experiments¹¹ indicate that cooling times as long as hours can inhibit crystallization, and this is comparable with the calculated cooling times of the expected lava flows.

If clathrates are present then they are stable in the presence of the normal conductive thermal environment within the satellite. Figure 3 shows the clathrate stability curves, below which a clathrate will decompose into water ice and gas. The geotherm does not cross these stability curves (except in the outermost centimetre of the satellite) but the $T\text{-}P$ path of the magma does, at a depth from 0.1 km to several kilometres, depending on the clathrate. An explosive event is possible: heat from the magma can provide sufficient energy to build up a gas pressure in excess of lithostatic, thereby 'blowing the lid', perhaps explaining the troughs on the satellites. Localized vents may also occur. Clathrates which incorporate much higher molecular weight species than CH_4 are possible²⁹ and it is conceivable that these could be a source of the dark material which may have resurfaced Iapetus. A more probable source is material dissolved in the $\text{NH}_3\text{-H}_2\text{O}$ magma and transported to the surface in volcanic events.

The observational tests of these ideas are difficult. For example, the IR spectrum of Rhea is dominated by water ice³⁰ but this does not argue against the presence of surficial NH_3 , which may not have a clear IR signature if it is in a glass. Ammonia may also be spatially localized or preferentially lost. The morphological consequences of 'superdykes' (large fissures filled with frozen $\text{NH}_3\text{-H}_2\text{O}$) are difficult to quantify, and much of the evidence of volcanism may have been obliterated by impacts. It is possible that the processes described here are more apparent on the surfaces of the uranian satellites, some of which may have more extended volcanism (because at least one, Titania, may be larger than Rhea) and may have undergone less obliteration by impact.

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Aerosols in equatorial Atlantic air: *n*-alkanes as a function of particle size

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Very little information is available about the marine, terrestrial or anthropogenic origin of the organic carbon associated with atmospheric particles. This origin can be determined by the analysis of lipid markers such as fatty acids^{1–3}, sterols⁴ or *n*-alkanes^{2,3,5,6}. We have undertaken studies of *n*-alkanes^{2,7} and polycyclic aromatic hydrocarbons (refs 8, 9, and M. J. Tissier, J.-C.M. and A.S. in preparation) in the marine atmosphere of the North and equatorial Atlantic and evaluate here the concentrations and distributions of *n*-alkanes as a function of particle size. This enables us to comment on the concentrations of particulate *n*-alkanes in the marine atmosphere, to compare the relationship between *n*-alkane and organic carbon concentrations as a function of particle size, and to determine the origins of these markers as a function of atmospheric particle size.

Aerosol samples were collected during the Romancap cruise of the R/V *Capricorne* in the western equatorial Atlantic, Gulf of Guinea (see Fig. 1 for map and sampling conditions). The cruise took place in May–June 1977, not during the Harmattan season (November–March), which brings high loads of terrestrial aerosols to that region. Very stable meteorological conditions prevailed during the cruise—moderate wind speed around 6 m s^{-1} , high insolation and no rain. However, the wind direction was SE–SSE during the first part of the cruise (sample RO imp 1) and changed to S–SSW for the second part. RO imp 2 was collected during the period when the wind direction changed, and RO imp 3 when the S–SSW direction was established. This latter direction being associated with a short distance from the coast (~ 300 km) leads to a direct continental influence on the second and third aerosol samples, as is confirmed by the measurement of radon daughter concentrations¹⁰ (Fig. 1), using the method developed by Lambert and Polian¹¹.

Aerosols were collected using a five-stage cascade impactor for large air volumes (Sierra 235). The aerosol collector was

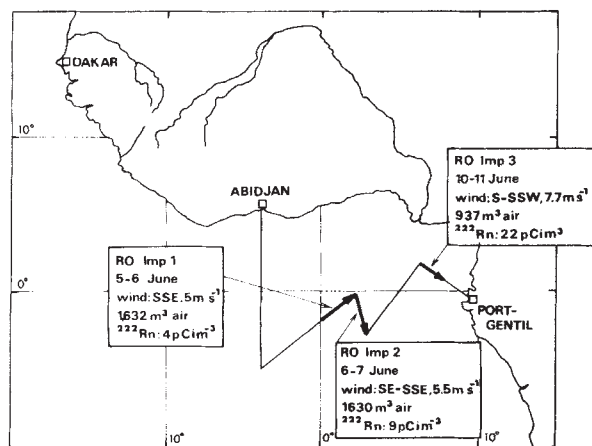


Fig. 1 Atmospheric sampling sites and conditions (wind direction and speed, air volume sampled and radon concentrations).

located on the bow of the ship at ~8 m above sea level. Samples were collected only when the ship was moving and the wind direction did not deviate more than 30° from the direction that the ship was heading. The impactor separated atmospheric particles into six size fractions, defined by their equivalent diameter (see Table 1). Air flow was 68 m³ h⁻¹ and collection times about 24 h leading to filtered air volumes of ~1,500 m³.

Before the cruise, the fibreglass filters were precleaned by extraction with chloroform in a Soxhlet for 24 h and kept in solvent-rinsed aluminium foil before use. Just after removal, filters were stored in glass tubes in a freezer. Organic carbon was determined on a portion of each filter using a Perkin-Elmer CHN analyser. Lipids were extracted from filters in a Soxhlet (24 h, benzene-methanol 1:1). After evaporation under reduced pressure, the extract was saponified (2 h, 2 M KOH-MeOH). Saturated hydrocarbons were separated from the non-

saponifiable extract by column chromatography. *n*-Alkanes were analysed by capillary gas chromatography using a Girdel 3000 gas chromatograph with flame ionization detector and a LDC 308 integrator, fitted with a glass, 25-m long, 0.25-mm internal diameter, wall-coated SE 52 column. The temperature was programmed from 120 to 270 °C at a rate of 4 °C min⁻¹ with helium as carrier gas at a flow of 2 ml min⁻¹. These conditions permit analysis of *n*-alkanes with 16–40 carbon atoms. External standards were used. Tests with standards in the same carbon range (C₁₆–C₄₀) indicated that the recovery of *n*-alkanes was better than 90%. Note here that data obtained for *n*-alkanes <C₂₀ may be underestimated, as they have a tendency to be revolatilized and thus lost during sampling especially at elevated ambient temperatures such as those encountered during the cruise¹². Blank samples taken during all sampling and laboratory work-up did not reveal detectable amounts of alkanes.

Table 1 gives *n*-alkane concentrations in the aerosols as a function of carbon number for different particle size ranges. Due to trace amounts of organic matter, RO imp 1 and RO imp 3 samples stages 3, 2, 1 and 5, 4 have been grouped together. Figure 2 plots the distributions of *n*-alkanes for the RO imp 2 sample for the six stages. The carbon preference index (CPI) is indicated for each sample at the end of Table 1. In the >C₂₀ range, *n*-alkanes of marine biological origin have CPI values in the range 1–1.5 (ref. 13). In contrast, cuticular waxes of higher plants are characterized by high values up to 15 of CPI^{14,15}. Thus, the CPI can be used to evaluate biogenic terrestrial contributions in marine samples, water or sediments. On Fig. 3, concentrations of organic carbon, *n*-alkanes and CPI values, are plotted as a function of the particle size distribution.

n-Alkane concentrations of aerosols varied in the range 6–13 ng m⁻³, as corroborated by values in the range 1.5–14 ng m⁻³ obtained during the same cruise using conventional aerosol samplers⁷. Note that these concentrations are comparable with other data: ~32 ng m⁻³ for tropical North Atlantic^{16,17}; 4–50 ng m⁻³ for tropical East Atlantic², 3–4 ng m⁻³ for the North

Table 1 *n*-Alkane concentrations (in ng m⁻³) as a function of particle size for RO imp 1, 2, 3 samples, Romancap cruise

Impactor stage	RO imp 1				RO imp 2							RO imp 3			
	F	5, 4	3, 2, 1	Total	F	5	4	3	2	1	Total	F	5, 4	3, 2, 1	Total
<i>n</i> -alkane															
<i>n</i> -C ₁₅							6				6			15	44
<i>n</i> -C ₁₆							12				12			18	53
<i>n</i> -C ₁₇	155			155	81		19				99	346	21	19	446
<i>n</i> -C ₁₈	144			144	104		28				132	297	10	26	395
<i>n</i> -C ₁₉	69			69	58		31				89	124	10	29	232
<i>n</i> -C ₂₀	46	2		50	23		31		3		57	49	17	41	205
<i>n</i> -C ₂₁	40	8	12	93	23		34		9		66	74	27	45	265
<i>n</i> -C ₂₂	109	15	23	208	46	16	62	6	25	3	159	297	131	49	705
<i>n</i> -C ₂₃	132	50	52	387	104	25	84	12	40	15	280	371	148	63	858
<i>n</i> -C ₂₄	132	93		535	127	29	108	43	56	28	391	347	155	92	934
<i>n</i> -C ₂₅	144	112	105	684	127	37	139	62	59	31	455	396	196	104	1,099
<i>n</i> -C ₂₆	178	124	159	903	150	37	146	74	65	34	506	421	191	118	1,157
<i>n</i> -C ₂₇	184	139	192	1,039	161	50	158	99	84	40	592	421	196	152	1,270
<i>n</i> -C ₂₈	178	135	198	1,044	138	58	139	99	74	46	555	322	112	97	839
<i>n</i> -C ₂₉	167	178	211	1,155	230	83	232	229	186	136	1,097	545	264	232	1,769
<i>n</i> -C ₃₀	86	116	126	696	81	29	96	68	31	19	323	149	61	55	436
<i>n</i> -C ₃₁	98	124	105	662	161	45	133	173	142	71	727	322	231	188	1,350
<i>n</i> -C ₃₂	52	66	50	332	46	12	56	43	25	12	195	49	37	45	261
<i>n</i> -C ₃₃	52	81	52	369	109	16	87	124	90	53	479	124	123	123	739
<i>n</i> -C ₃₄	11	12	7	56	6	4	25	12	3	3	53		15	31	123
<i>n</i> -C ₃₅		12	6	42	58		19		6	9	92		19	50	188
<i>n</i> -C ₃₆							12		1		14		10	10	49
<i>n</i> -C ₃₇							6				6				
Total	1,977	1,268	1,370	8,623	1,831	442	1,664	1,003	900	502	6,342	4,655	1,976	1,604	13,419
CPI	1.06	1.26	1.16	1.17	1.58	1.38	1.36	2.02	2.20	2.45	1.71	1.40	1.68	1.87	1.65

Equivalent diameters (d_{eq}) of aerosols corresponding to the impactor stages are: F, final filter: $0.003 < d_{eq} < 0.5 \mu\text{m}$; stage 5: $0.5 < d_{eq} < 0.96 \mu\text{m}$; stage 4: $0.96 < d_{eq} < 1.5 \mu\text{m}$; stage 3: $1.5 < d_{eq} < 3 \mu\text{m}$; stage 2: $3 < d_{eq} < 7.2 \mu\text{m}$; stage 1: $d_{eq} > 7.2 \mu\text{m}$.

The carbon preference index (CPI) is calculated over the range n -C₂₀ to n -C₃₆:

$$\text{CPI} = \frac{1}{2} \left[\frac{\sum_{n=21}^{n=35} C_{\text{odd}}}{\sum_{n=20}^{n=34} C_{\text{even}}} + \frac{\sum_{n=21}^{n=35} C_{\text{odd}}}{\sum_{n=22}^{n=36} C_{\text{even}}} \right]$$

C_{odd} and C_{even} are odd and even carbon numbered alkanes respectively.

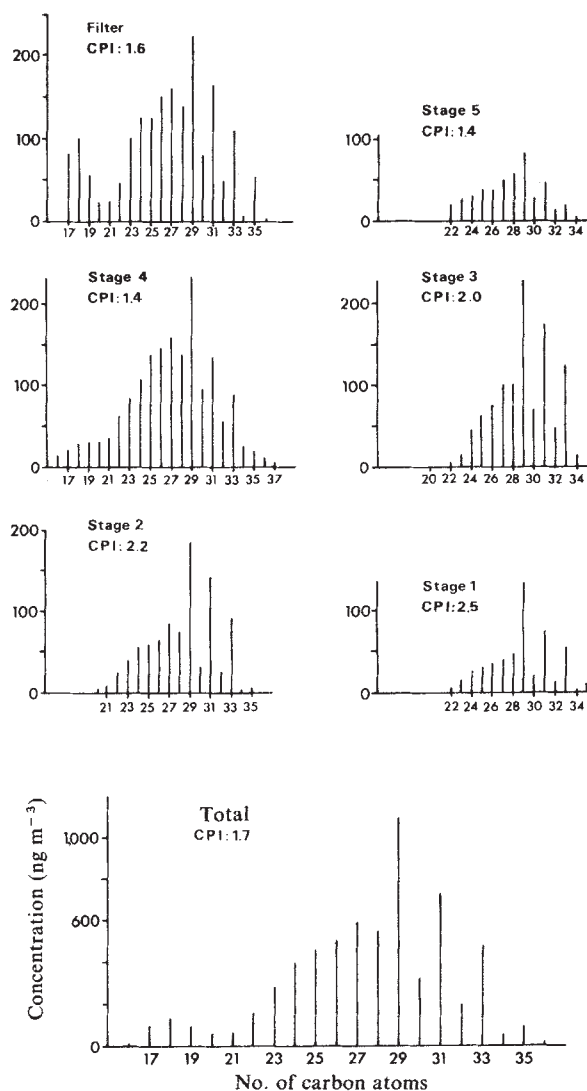


Fig. 2 Reconstructed chromatograms of *n*-alkanes of the six stages of the impactor for RO imp 2 sample.

Atlantic, Loophead Ireland⁵, 3–4 ng m⁻³ for the Indian Ocean⁶. These first results concerning the equatorial Atlantic are useful for large-scale budgeting of continent-ocean exchanges of hydrocarbons because the marine air of the Guinea gulf also seems representative of the Atlantic. The Atlantic also seems to have considerably higher atmospheric *n*-alkane concentrations than does the tropical North Pacific, where values in the range 0.02–0.16 ng m⁻³ were reported by Gagosian *et al.*³. High levels of atmospheric organic matter for the Atlantic are also indicated by organic carbon concentrations in the range 1.2–2.3 ng m⁻³ (Fig. 3). There is a clear correlation between high levels of *n*-alkanes and of organic carbon.

The distribution of organic carbon as a function of particle size shows that most of the aerosol mass is associated with the fine particles <1 µm. This observation agrees with previous results^{18,19}, but the relationship between organic carbon and *n*-alkane concentrations is different. For *n*-alkanes, if the major part is associated with finer particles, 60% is in the range <1 µm, which is less than for the organic carbon, 80%. Organic carbon presents a distribution as a function of particle size very different from that of sodium, which is carried primarily by particles with a diameter in the range 3–4 µm (ref. 18). The difference between organic carbon and sodium as a function of particle size can be explained by two processes:

(1) The direct ejection from the ocean of carbon rich particles (<1 µm) containing no sodium. As we have recently demonstrated using organic markers such as sterols⁴ and fatty acids⁷,

one of the major mechanisms in sea-air exchange is the ejection of particles from the sea-surface microlayer by bubble breaking. This direct input of particulate matter is strongly supported by data of Johnson and Cooke²⁰ and illustrates the importance of particles associated with bubbles in seawater.

(2) The ejection of seawater droplets (jet and film drops) which lead after desiccation to medium size (~3 µm) salted particles.

The distribution of total particulate *n*-alkanes shows common characteristics which clearly indicate a marine origin: regular distribution of *n*-alkanes in the range *n*-C₂₀ to *n*-C₂₅, presence of medium molecular weight compounds in the range *n*-C₁₅ to *n*-C₁₉, absence of the unresolved components (hump) and very low concentrations of pristane and phytane typical of petroleum contaminants²¹. Another contribution is indicated by the predominance of odd carbon number *n*-alkanes in the range *n*-C₂₅ to *n*-C₃₇ in *n*-alkane distributions (Fig. 2). This terrestrial contribution is slight in the first sample (CPI = 1.2), more important in samples RO imp 2 and RO imp 3 (CPI = 1.7 and 1.6). This interpretation is confirmed by ²²²Rn measurements (see Fig. 1).

Another interesting feature is the relationship between CPI values and particle size distributions as shown in Fig. 3. It can be seen that terrestrial inputs are carried by larger particles. Effectively our marine reference RO imp 1 shows CPI values close to unity for all particle sizes. The CPI increases from 1.5 for particles <1 µm to 1.9 (RO imp 2) and 2.5 (RO imp 3) for particles >1 µm. By comparison, Thompson and Eglinton²² analysing *n*-alkanes of a lacustrine sediment observed that, as for aerosols, the CPI values decreased with decreasing grain size. Generally the direct input of aerosols to ocean bottom

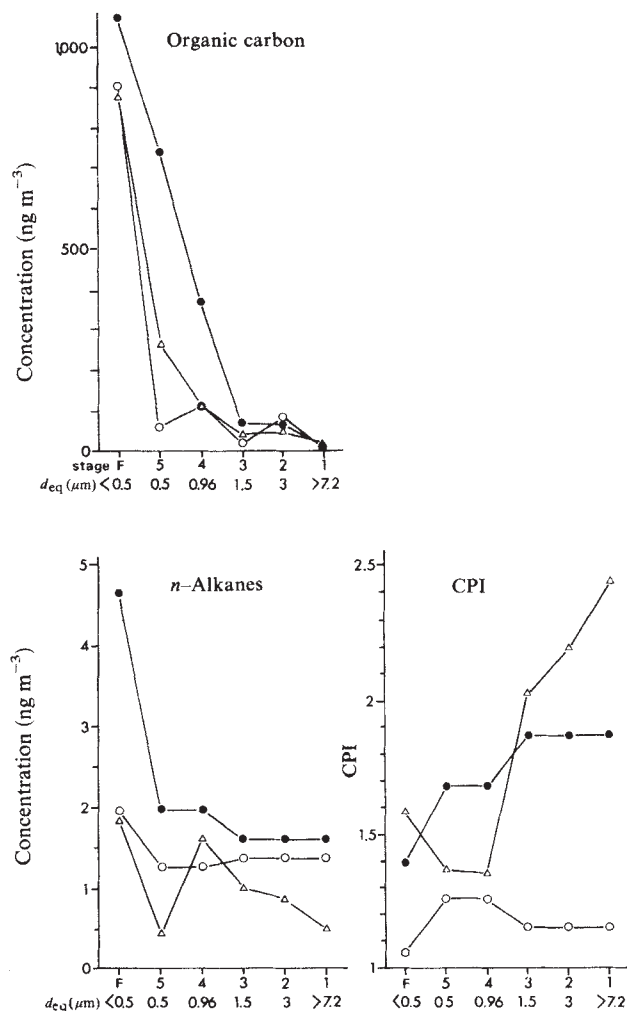


Fig. 3 Organic carbon and *n*-alkane concentrations and CPI values as a function of particle size, Romancap cruise. ○, RO Imp 1; △, RO Imp 2; ●, RO Imp 3.

sediments is the probable explanation for the surprisingly high amounts of terrestrial material in sediments far removed from land.

Our results indicate a terrestrial origin for most of the atmospheric material $>1\ \mu\text{m}$. However, these results cannot be generalized to other geographical areas. For example, in the central Pacific, which is very poor in atmospheric *n*-alkanes (10–100 times less than for the Atlantic), Chesselet *et al.*¹⁹ found, using a $\delta^{13}\text{C}$ technique, that larger size particles ($>1\ \mu\text{m}$) were principally marine.

An interesting comparison can be made between our data and those of Van Vaeck and Van Cauwenberghe²³ related to an analysis of *n*-alkanes as a function of particle size in the atmosphere of the suburban zone near Anvers, Netherlands. They showed that the increasing relative importance of odd carbon number *n*-alkanes was related to the size of particulates. This observation corroborates our results, especially because their *n*-alkane distribution for particles $<1\ \mu\text{m}$ indicated a mixed influence, terrestrial and marine. Another point supports this result. Simoneit²⁴, analysing aeolian dusts ($>2\ \mu\text{m}$) over the entire Atlantic found that these particles were essentially of terrestrial origin, with high values of CPI from 2 to 10. All these data demonstrate that for the equatorial Atlantic there is a lack of coupling between marine and terrestrial aerosols; fine particles are of marine origin and larger size particles ($>1\ \mu\text{m}$) are largely derived from continental sources.

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Black smokers fuelled by freezing magma

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If ocean floor hot spring activity is to continue long enough to produce a large massive sulphide deposit, heat must be stripped by the circulating water from a store within the ocean crust. We argue here that this heat store can only be a magma chamber, and that production of a large sulphide deposit requires very rapid crystallization of large volumes of magma ($\sim 1\ \text{km}^3$ per 100 yr for several hundred years). The effects of such rapid solidification should be apparent in the gabbroic components of ophiolite complexes, as well as in plutons in other environments where large high temperature massive sulphides are formed.

The black smokers described from the crest of the East Pacific Rise at 21°N ^{1–4} are very hot springs discharging water blackened by finely divided iron, zinc and copper sulphides. They occur very close to the spreading axis and are the discharge points of single pass thermosyphon hydrothermal systems fed by cold seawater. Recent observations of the springs and their associated deposits² have established the temperature (350–390 °C), the linear flow rate ($1\text{--}5\ \text{m s}^{-1}$), orifice size ($\sim 0.4\ \text{m}$ diameter) and composition of the emerging hot waters. From these figures, the power output of a single vent (relative to an input of cold seawater) is $\sim 3 \times 10^5\ \text{kW}$. It has been shown² that this rate of power output far exceeds the rate of supply of heat to the mid-ocean ridge crest from the mantle, and that the operation of the black smokers must thus be episodic.

Massive sulphide deposits ranging up to 15–20 million tons in size which are found in ophiolites, slices of ocean crust emplaced above sea level, appear to have formed from sea floor hot springs at 350 °C (ref. 5) close to the ancient spreading axis⁶. The parallel with the black smokers is thus excellent. The observed content of iron in the black smoker fluid (110 p.p.m.)⁴ allows calculation of the time necessary for one black smoker to form a 3 million ton massive sulphide deposit of the kind found in the Troodos ophiolite in Cyprus (2,500 yr). If a cluster of, say, three black smokers is responsible, then the time would be reduced to 800 yr. In any event, the necessary output of heat for such a deposit would be about $2.5 \times 10^{19}\ \text{J}$. Where can this source of heat be?

The problem is twofold. One is to supply the total amount of heat necessary at a rate faster than it emerges from the mantle. This requires some sort of store where heat can be held back until it is tapped by the black smoker system. The other is to extract the heat from the store at a high enough temperature over the necessary period of time: this is a particularly severe constraint. Two kinds of store can be readily envisaged. One is hot rock, the other is magma: we now look at these in turn.

To do this we use a semi-analytical computer model of hydrothermal circulation in the ocean crust which we describe in detail elsewhere⁷. In the model (Fig. 1) the circulation is confined to a single fault zone, envisaged as a newly-formed ridge-crest parallel fault such as those with which ore-deposits in Cyprus are associated, and along which sea-floor hot springs are aligned^{3,8,9}. We believe that these faults act as highly permeable pathways that localize hydrothermal heat transport in the ocean crust¹⁰. The flow is modelled as an open-loop thermosyphon, which exchanges heat across the fault walls with a semi-infinite solid on each side by transient one-dimensional conduction. Subsidiary porous-medium convection can be simulated by increasing the apparent thermal conductivity of the rock. The fault can be suddenly opened to any desired depth with an inflow of water at 0 °C in a medium of arbitrarily determined vertical thermal structure, and can then be propagated downwards at any suitable rate through hot rock. Heat can be supplied if desired to the base of the fault to simulate latent heat extraction from solidifying magma. In all of our runs the model was tuned to black smoker conditions by setting the mass flow rate to $160\ \text{kg s}^{-1}$ and the upflow velocity to $2\ \text{m s}^{-1}$. Because downflow velocity is related directly to the length of the recharge zone along the fault because of continuity of mass, a change in the downflow velocity has the effect of changing the water/rock ratio in terms of the volumes involved in heat exchange.

The first runs of the model examine the extraction of heat from the upper kilometre of ocean crust at a ridge crest. Here the initial rock temperature increases steadily downwards to 1,000 °C and the fault opens suddenly to 500 m and then propagates steadily downwards at $\sim 7\ \text{m yr}^{-1}$ (ref. 11) to 1 km. Two downflow velocities were used, $10^{-3}\ \text{m s}^{-1}$, which corresponds to a recharge zone 1 km long, and $10^{-4}\ \text{m s}^{-1}$, corresponding to a 10-km recharge zone. The results obtained (Fig. 2) show that a 1-km recharge zone cannot supply enough heat at a high enough temperature. The 10-km recharge zone can

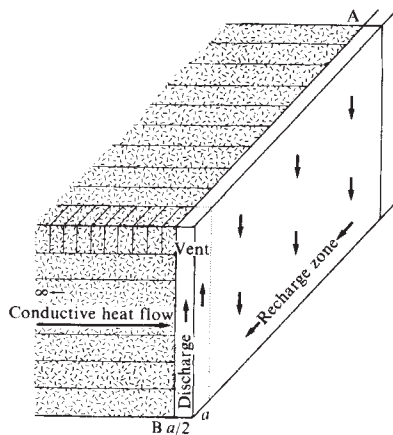


Fig. 1 Geometry of the model. Flow is confined to the constant width slot formed by the fracture. Only the half-width of the fault is shown, a mirror image of the system is required for the full width. The length of the recharge zone along the fault is Ya where Y is the upflow speed/downflow speed and a , the full fault width, assuming a square vent. The recharge and discharge limbs are thermally independent. The rock is treated as two stacks of uniform semi-infinite blocks each with a different initial temperature and insulated on all faces except those perpendicular to the conductive heat flow. The temperature profile in each block and the heat flux across the water-rock interface (the plane AB) is obtained by using the one-dimensional transient heat conduction equation for a semi-infinite solid with a prescribed boundary temperature²¹. The computation is cyclical to allow the boundary conditions to be changed after each cycle. The heat exchange is computed for each block of rock in turn starting at the ocean floor and working through the loop. For each time step a thermal profile in the block of rock and the water temperature in the fault at that level are obtained. The water temperature in the previous block is used as the inflowing water temperature to the next block for that cycle, so that the water is passed through the loop exchanging heat as it progresses. When the model is tuned to black smoker conditions the fluid dynamics of the system require an upflow consisting of four rough tubes with diameters of half the vent width rather than a void slot.

supply enough heat to run one black smoker for nearly 200 yr, but this recharge zone length implies a vent spacing an order of magnitude greater than that observed on the East Pacific Rise², and a convection cell with a 10:1 aspect ratio. The long recharge zone can thus be rejected, and so can the possibility of fuelling long-lived black smokers from the upper kilometre of the crust.

The next runs of the model examined whether propagation of the crack to greater depths in hot crust could provide enough high-grade heat. The lower 5 km of the 6 km thick crust were taken to be at 1,000 °C, as though a magma chamber had just finished crystallizing. The fault propagated downwards through this lower 5 km layer at about 10 m yr⁻¹. Again it was impossible to obtain a long-lived black smoker. In these conditions, with a vent spacing of 1 km, the vented water remained close to 200 °C for several hundred years, but did not reach 350 °C after the first 2–3 months. Increasing the thermal conductivity of the rock to simulate subsidiary porous medium convection increased temperatures somewhat, but the gain at depth was largely cancelled by losses in the rising limb. These runs showed that a large ore deposit could not be fuelled from hot rock within the framework of the model, essentially because the rate of conduction of heat is too slow to maintain the necessary high temperatures in the circulating fluids, even though an apparently abundant source of heat is available. The only model we know which can produce large amounts of water at high temperature from hot rock alone is that of Lister¹², in which a network of fine cracks is allowed to propagate downwards several kilometres into hot rock, extracting heat into circulating water. We find the framework of this model unconvincing on several grounds, principally because it requires a much greater degree of water-rock interaction than can be seen in the gabbros of ophiolite complexes^{13,14}, which represent the level from which the heat would need to be extracted.

Magma as a heatstore avoids the problem of conduction in solid rock, because heat can be convected through the magma

to a thin conductive boundary layer separating the magma from the hydrothermal circulation. Consideration of the overall geology shows that enough heat should be available in this way. Figure 3 shows a cross-section of a mid-ocean ridge with a magma chamber, based on evidence from the ocean floor^{15,16} and from ophiolites¹⁷. In the mature crust, nearly 4 km of cumulates underlie 1 km of dykes and 1 km of lavas. These cumulates must form completely during the time it takes for the crust to spread half the width of the magma chamber, and, as they form, latent heat of crystallization is released in the magma. This latent heat is a potential source of heat for the black smokers.

How much latent heat would be required? The total power output of a cluster of three black smokers is 9×10^5 kW. Some of this heat would be derived from hot rock, and some lost to cold rock on the ascent. On running our model with a heat input at the base sufficient to simulate black smoker output, not more than 10% of the heat originates from the rock, and the other 90%+ had to be input from below, in this case from latent heat.

We now consider the rate at which magma must solidify to supply the heat for a cluster of three black smokers. Taking the latent heat of solidification of basalt as 4×10^5 J kg⁻¹ and a magma density of 2,700 kg m⁻³, this requires the solidification of 2.5×10^7 m³ of magma yr⁻¹. If the three black smokers are to run for the 800 yr necessary for them to produce a 3 million ton ore deposit then this would require the latent heat from 20 km³ of magma.

Compare this with the steady-state rate of supply of latent heat for a 1-km stretch of ridge crest. For a half-spreading rate of n cm yr⁻¹, the solidification rate is $n \times 8 \times 10^4$ m³ yr⁻¹, and the rate of supply of latent heat thus $n \times 3.4 \times 10^3$ kW. At a spreading rate of 3 cm yr⁻¹, this would provide enough heat to power a group of three black smokers provided that the heat extraction is episodic and operates for only 1% of the time. But is this fraction of the time long enough to form large ore deposits? If the hydrothermal circulation is localized along the ridge-crest parallel faults as suggested above, then a fresh episode of hydrothermal circulation would be expected each time a new fault formed. Since major faults of this kind are spaced 1–2 km apart independently of spreading rate^{9,18}, the time interval between successive faults is $\sim 1/10n$ Myr. During this time the cluster of vents could operate for:

$$\frac{n \times 8 \times 10^4}{2.5 \times 10^7} \times \frac{10^6}{10n} = 320 \text{ yr}$$

This time is long enough to produce a 1 million ton ore deposit, and is independent of spreading rate. Ore deposits of 10 million tons or more, such as are found in some ophiolite complexes, would require rather special conditions, such as an unusual influx of magma, collection of heat from further along the ridge crest, or a longer delay between circulation episodes, but are well within the possible range of variation.

Not all episodes of hydrothermal circulation would result in ore deposits. We found it necessary in constructing our model⁷

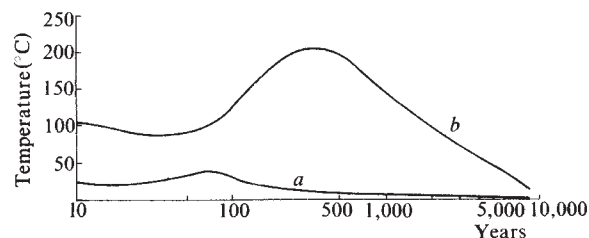


Fig. 2 Plot of vented water temperatures as a function of time. Recharge flow speed, 10^{-3} m s⁻¹; upflow speed, 2 m s⁻¹; length of recharge zone, 1 km. *a*, Circulation through 1 km of rock with initial thermal gradient 0–1,000 °C overlying magma chamber. Fault opened initially to 470 m depth and propagated downwards at ~ 7 m yr⁻¹. *b*, Circulation through 6 km of rock with initial thermal gradient 0–1,000 °C in 1 km above 5 km at 1,000 °C, assuming no magma chamber present. Fault opened initially to 1 km and propagated downwards at ~ 10 m yr⁻¹.

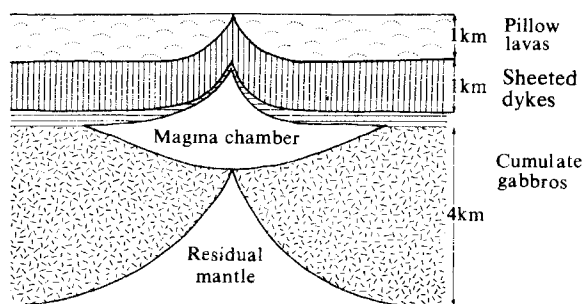


Fig. 3 Axial structure of a mid-ocean ridge.

to throttle back the circulation by modelling the upflow as four narrow rough pipes to allow the necessary high temperatures to be reached. If the fault were more open than this, the rate of flow would be such that only warm water would emerge from the vent, without the capacity to make a large ore deposit, because of the large drop in solubility of iron in water as temperature falls¹⁹. Such episodes of cooler circulation are one reason why large ore deposits would appear to be rather rare on the ocean floor²⁰. However, in the right hydrogeological conditions we envisage a situation where a new fault reaches to the plastic roof zone of the magma chamber which acts as a thin conductive boundary layer. The hydrothermal circulation in the fault will give a cold line on the roof of the magma chamber causing very active convection in the chamber, which will bring the released latent heat rapidly to the cold line heat sink where it can be extracted by the circulating water. As this takes place, the emerging hot water will precipitate massive sulphide ore at a rate of about 1 million tons for each 7 km³ of magma solidified. Alternating with these very active phases of circulation will be periods of no hydrothermal circulation, during which the magma chamber will grow and the store of latent heat will be replenished.

The effects of such rapid and spasmodic growth of cumulates in mid-ocean ridge magma chambers should be evident in the gabbro sequences of ophiolite complexes. The characteristic high-level gabbros of ophiolites, with their rapidly-changing grain size and hornblende-bearing pegmatitic patches probably represent the conductive boundary layer on the roof of the magma chamber, which has advanced downwards during cooling to produce a complex structure containing a history of prolonged cooling. In the cumulate gabbro below, the rapid sedimentation rate of crystals should show up as a sequence of cumulate dominated by closed-system fractionation, because replenishment of magma during such a short time is unlikely. Zoning would probably have been removed by sub-solidus diffusion in the hot cumulates, but the necessary intensive convective circulation in the chamber must have left a recognizable sedimentary record.

The same principles we have proposed for the ocean crust should also be applicable to all large high-temperature massive sulphide deposits, wherever they occur, such as the Kuroko deposits of volcanic arc regions. Hot rock alone is unlikely to have been able to supply enough heat, and latent heat from magma chambers must have been important in their development. Approximately the same equation between magma crystallized and ore produced (7 km³ per million tons) should apply, with the result that production of a large ore deposit should result in a substantial degree of crystallization of a pluton within a short space of time. Such events would show a clear record in the pluton, and also in the volcanic pile associated with it, where it may be possible to use lava stratigraphy to predict horizons at which substantial ore deposits could be found, as has been suggested on empirical grounds in the past. Recognition of the appropriate effect could result in a much improved success rate in prospecting for massive sulphide deposits in volcanic terrains, and as such clearly deserves following up.

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Gulf of Aden axial magnetic anomaly and the Curie temperature isotherm

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The main features of magnetic anomalies over ocean ridges have been explained¹ as a corollary of seafloor spreading and geomagnetic reversals. Oceanic crust is formed in a narrow region, becoming magnetized in the direction of the Earth's magnetic field as its temperature falls through the Curie point of the magnetic minerals present. The Gulf of Aden was one of the first places where reversely magnetized sea floor was recognized². The seafloor spreading direction and latitude are such that the anomaly due to normal magnetization is negative and slightly skewed. Positive anomalies were also observed suggesting the presence of reverse magnetization. A short wavelength magnetic anomaly which frequently occurs superimposed on the axial magnetic anomaly in the Gulf of Aden is now described. Various interpretations are considered; the preferred involves a dramatic shallowing of the Curie temperature isotherm close to the seafloor spreading centre. The interpretation has implications for models of the generation of oceanic lithosphere and for locating possible geothermal areas in rifted regions.

Magnetic profiles across the Gulf of Aden show impressive seafloor spreading anomalies, the axial anomaly reaching –2,000 nT in the west and –1,000 nT in the east. The spreading rate is ~1 cm yr⁻¹ per limb^{3–5}. A selection of profiles across the ridge axis is shown in Fig. 1a and their locations in Fig. 1b. All profiles were recorded with proton magnetometers towed astern. The water depths for the profiles in the western Gulf of Aden range from 1,000 to 1,500 m and for the profiles in the eastern Gulf of Aden from 2,000 to 3,500 m.

A feature often observed is a short wavelength positive anomaly superimposed and centrally disposed on the axial negative anomaly. The feature is more common on profiles in the western Gulf of Aden (see profiles A, B, B', E, F, G, H, J Fig. 1a) but is also seen in the east (see profile 4, Fig. 1a). The frequent occurrence of the feature merits a comprehensive study.

Seafloor spreading models were constructed and synthetic magnetic profiles computed using the method of Talwani and Heirtzler⁶ and the reversal time scale of La-Breque *et al.*⁷. Magnetizations were chosen to match approximately the amplitudes of the observed anomalies and as frequently noted, a greater magnetization is required for the crust beneath the axial

anomaly than away from it. The short wavelength positive anomaly superimposed on the axial negative anomaly is not reproduced in the synthetic profiles derived from the usual normally and reversely magnetized blocks.

First, it was necessary to investigate whether the small central positive anomaly might be due to rugged bathymetry. Synthetic profiles 1 in Fig. 2 show the effects of incorporating the bathymetry. The frequency content of the observed profiles is reproduced quite well for a magnetized layer with thickness ~ 2 km. Short wavelength features due to bathymetry are generated but they bear no correspondence to those observed. It is clear that the central short wavelength positive anomaly is not a bathymetric effect.

A second possibility is that the anomaly is due to recent variations in the intensity of the geomagnetic field^{8,9} that is secular variation and short period reversals (excursions) within the Brunhes epoch. This seems unlikely. The anomalies are 5–9 km wide and if we assume a constant spreading rate of 1 cm yr^{-1} over the whole of the past 5 Myr (ref. 5), this represents 250,000–450,000 yr which is many orders of magnitude too large. The largest excursion (Blake) is thought to be at most 13,000 yr and any others much smaller¹⁰. Further, the anomaly is not observed on all profiles, nor is it observed over faster spreading centres such as the East Pacific Rise. This suggests that it is not related to the tape recorder aspect of seafloor spreading but rather to some other physical phenomenon at the spreading centre.

The explanation suggested here is that the short wavelength positive anomaly superimposed on the axial negative anomaly is due to the magnetized layer being thin over the spreading centre. This implies that the Curie temperature isotherm shallows rapidly at the spreading centre. Figure 2 shows computations based on this interpretation for profiles F, B' (western Gulf of Aden) and 4 (eastern Gulf of Aden). A zone of zero magnetization (shaded) was introduced at the base of the magnetized crust at the spreading centre and its shape altered until a reasonable fit between synthetic (profiles 2 in Fig. 2) and observed profiles was obtained. The zones of zero magnetization in the models for profiles F and B' (Fig. 2a, b) are 3 km wide at their bases, tapering to 2 km wide at their tops, and extend to

0.8 km beneath the valley floor. The zone of zero magnetization in the model for profile 4 (Fig. 2c) is wider, being 5.5 km at its base, tapering to 3.5 km at its top and extending to 0.4 km beneath the valley floor.

The models are, of course, subject to the usual non-uniqueness of interpretations of potential fields. For example, if a thinner, more highly magnetized layer is assumed^{11,12}, the top of the zone of zero magnetization would reach close to the top of the magnetized layer. Different assumptions concerning the intensity of magnetization with depth would also affect the vertical extent of the zone of zero magnetization. However, the horizontal extent of the unmagnetized zone would require little modification.

The models and these considerations suggest that the Curie temperature isotherm is <0.8 km deep at the spreading centre. This would place it within seismic layer 2 where the crust is probably composed of basalts. The minerals responsible for the magnetization of fresh basalts dredged from the sea floor close to spreading centres are titanomagnetites. These have Curie temperatures¹³ of $\sim 165^\circ\text{C}$. The zone of zero magnetization must pass through the deeper oceanic crust, that is seismic layer 3 which is probably composed of gabbros. These have much higher Curie temperatures of about 550°C probably due to the presence of magnetites^{14,15}. This makes the estimation of the shape of the Curie temperature isotherm (demarcating the zone of zero magnetization) very complex.

Aeromagnetic profiles for the Reykjanes Ridge¹⁶ show a large simple positive anomaly over the ridge crest. Centrally disposed on the axial anomaly is a short wavelength negative anomaly. A rapid shallowing of the Curie temperature isotherm as the spreading centre is approached could also explain this.

So far, short wavelength anomalies superimposed on and having opposite sign to the axial anomaly have been discussed. The opposite observation has also been made for ridge crests in various parts of the world^{17–20} where short wavelength features superimposed and centrally disposed on the axial anomaly have the same sign. The explanation offered^{17–20} is that a narrow region of enhanced magnetization occurs in the vicinity of the spreading centre. There are profiles crossing the Gulf of Aden ridge crest which show such features and which could be explained in this way. The part of the oceanic crust close to the spreading centre which is more intensely magnetized is most likely to be the uppermost part where extrusives are quench cooled. The superimposed magnetic anomaly is then the result of a magnetization contrast near the top of the magnetized layer. It might be possible in certain circumstances to identify

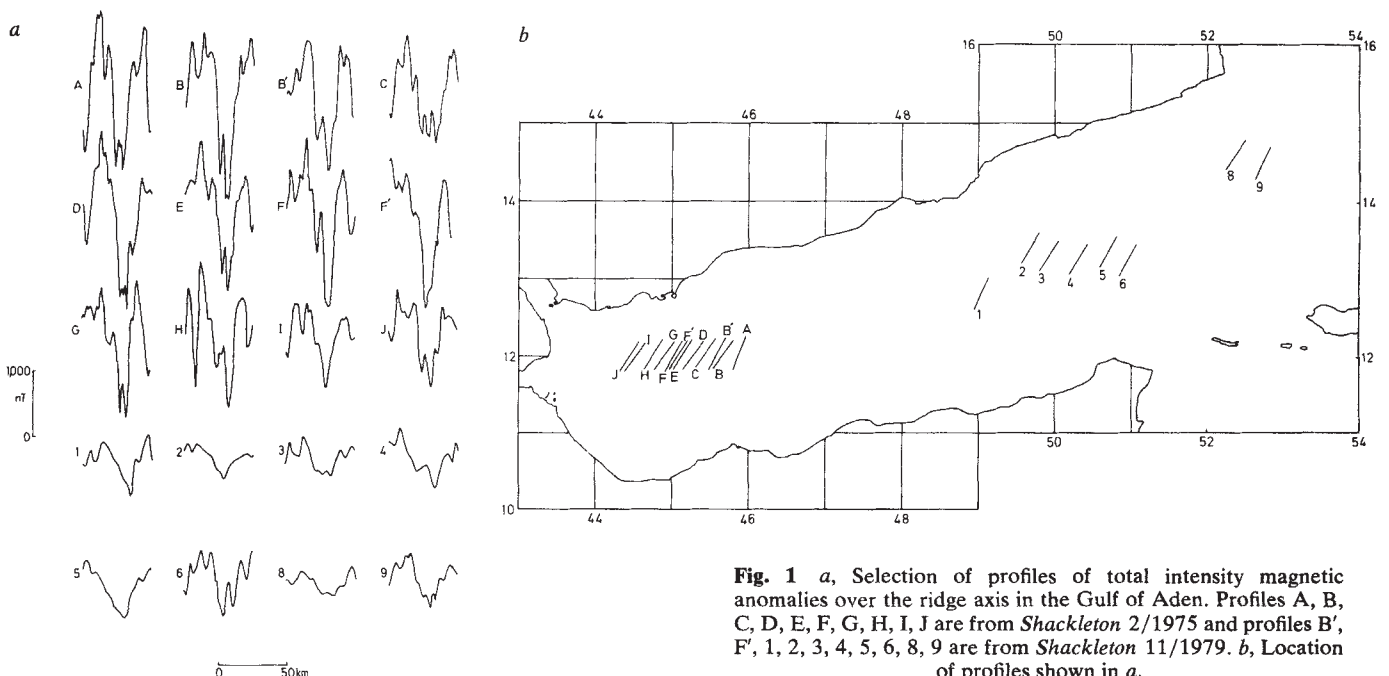


Fig. 1 a, Selection of profiles of total intensity magnetic anomalies over the ridge axis in the Gulf of Aden. Profiles A, B, C, D, E, F, G, H, I, J are from Shackleton 2/1975 and profiles B', F', 1, 2, 3, 4, 5, 6, 8, 9 are from Shackleton 11/1979. b, Location of profiles shown in a.

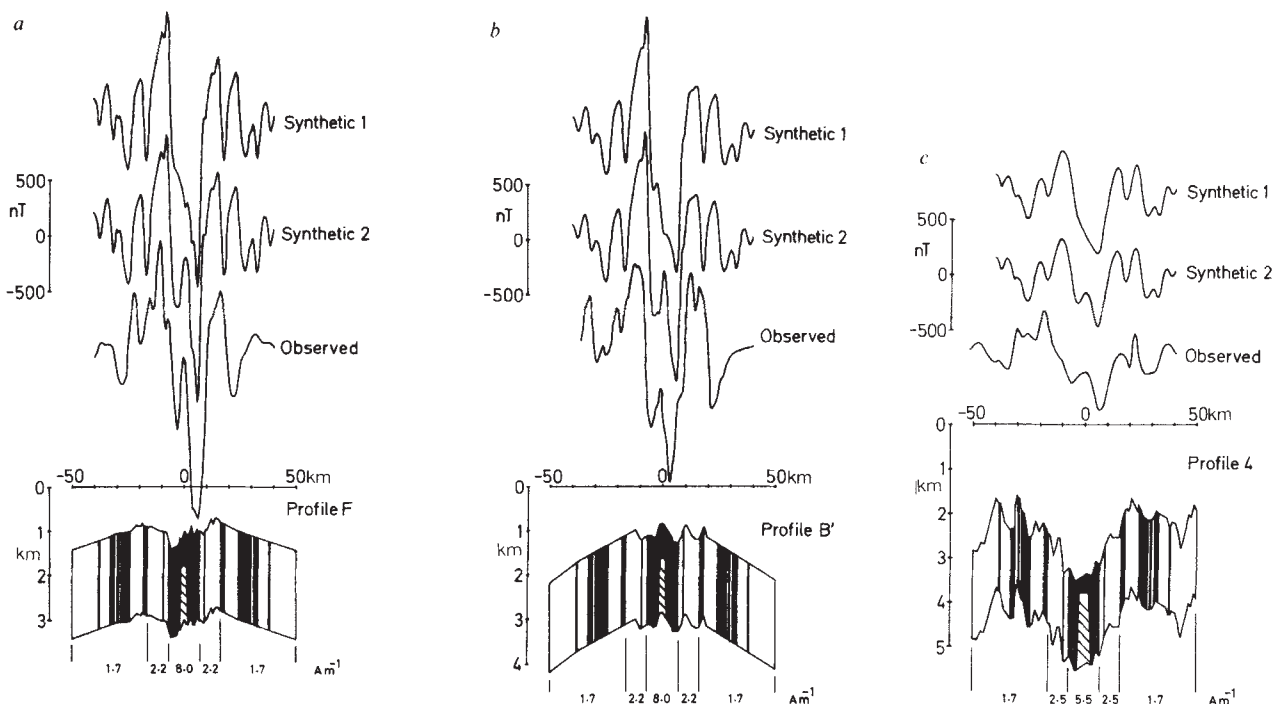


Fig. 2 Normally and reversely magnetized crust (geomagnetic reversal time scale of La-Breque *et al.*⁷ seafloor spreading rate of 1 cm yr^{-1}) represented by black and white blocks respectively. The central hatched block is normally magnetized like the central black block in the computation of synthetic profile 1 and has zero magnetization in the computation of synthetic profile 2. Vertical exaggeration 20:1. *a*, Profile F. *b*, Profile B'. *c*, Profile 4.

anomalies due to both effects, if both are present, as they occur at different depths and hence should have different wavelength content. The two effects might be more easily separated using profiles obtained nearer to the sea floor.

A possible mechanism for producing a rapid shallowing of the Curie temperature isotherm close to the spreading centre is the occasional passage of hot magma through the entire thickness of the lithosphere in this region. The positions of the inferred hot intrusions do not deviate by $\geq 1 \text{ km}$ from the mean position of the axis of spreading.

It might be thought that the larger amplitude of the axial magnetic anomaly in the western Gulf of Aden than in the east is due to the sea floor in the west being shallower than in the east. If the profiles are upward continued to the same height a large discrepancy in amplitude remains. However, the upward continued profiles in the west show a severe attenuation of the short wavelength positive anomaly superimposed on the axial anomaly. Hence the shoaling from east to west might explain the more common occurrence of the superimposed positive anomaly in the west. This emphasizes the value of obtaining magnetic profiles closer to the sea floor and at a constant height above it.

Short wavelength anomalies superimposed on and having the same sign as the axial anomaly have been used to identify the positions of recent activity on spreading centres¹⁸⁻¹⁹. Short wavelength anomalies superimposed on but with opposite sign to the axial anomaly where observed might serve the same purpose.

If the interpretation of the short wavelength positive magnetic anomaly superimposed and centrally disposed on the negative axial anomaly is correct, it has interesting implications for magnetic and thermal models of the oceanic crust. It supports the idea that at depth the boundary between normally and reversely magnetized blocks is sloping rather than vertical²¹⁻²³. Maximum depth determinations of the Curie temperature isotherm at the spreading centre would provide a useful constraint on theoretical thermal models of ocean ridge crests.

The fact that some magnetic profiles in the western Gulf of Aden do not show a short wavelength positive anomaly over the spreading centre might reflect the lack of continuity of a higher

temperature region along the axis and the episodicity of the thermal structure of spreading centres. This suggests that the presence of the anomaly might be an indicator of regions suitable for geothermal exploration associated with spreading centres. An example is seen in Iceland where the Krafla and Namafjall geothermal areas are associated with negative magnetic anomalies²⁴. Here the magnetic latitude is such that the anomaly over the spreading centre is positive. These are of similar width to those observed in the Gulf of Aden. Thus the presence of magnetic anomalies of opposite sign superimposed on the axial anomaly might indicate a shallower Curie point isotherm and hence the presence of a geothermal area.

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³⁹Ar/⁴⁰Ar dating of the trans-Himalayan calc-alkaline magmatism of southern Tibet

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The age of the trans-Himalayan calc-alkaline magmatism has not been clearly established: on the basis of geological control, it is possible to infer a pre-late Cretaceous age for the plutonites at the southern edge of the batholith belt, and a late Cretaceous or younger age for the lava series on the top and/or to the north of the plutons. K-Ar radiometric data previously obtained by Chinese groups agree with these geological ages. The ³⁹Ar-⁴⁰Ar method has been used to analyse more accurately some of the rocks which have been sampled during the joint French-Chinese field work in southern Tibet in the summer of 1980: the incremental degassing of argon gives generally more information about the thermal history of the sample. Using this method we now show that the earlier basic plutons of the southern edge of the trans-Himalayan belt have ages of 90–110 Myr (Albian to Cenomanian), whereas the lavas of the upper unconformable series are clearly younger (60 Myr, Palaeocene). The Ladakh and Kohistan magmatic belts further west are probable equivalents of the trans-Himalaya, north of the Indus suture. Their precise ages are also poorly known: Orbitolinas of middle Cretaceous (Aptian) age have been found in the Drosch-Yasin series which lie unconformably on dioritic plutons in the northern edge of the belt. The few radiometric data that are available give ages in fairly good agreement with those we have obtained in the trans-Himalaya. So we conclude that a subduction zone has been active during the Cretaceous between India and Asia. The only outstanding question is whether this active margin was, at that time, close to India, or to Asia, or somewhere in between.

The trans-Himalayan (Kangdese) plutonic belt, north of the Tsangpo ophiolitic suture, can be interpreted, together with the associated calc-alkaline volcanics of Middle Cretaceous to Lower Tertiary age, as the product of partial melting in a North dipping Benioff zone, before the emplacement of ophiolites and continental collision along the Tsangpo suture¹⁻³.

Geological evidence for the age of emplacement of these calc-alkaline magmatic rocks come from: the Xigaze series of Upper Cretaceous age lying unconformably on some of the Kangdese dioritic batholiths; interbedding of calc-alkaline lavas and tuffs (mainly andesitic and dacitic) in the Middle-Upper Cretaceous Takena series north of the batholith, and in some places in the Xigaze group itself; thick andesitic or other intermediate to acid calc-alkaline series in the Lingzizong formation, lying unconformably on top of the Takena series. Marine fossils of Palaeocene age⁴ have been found in Lingzhu area, north of Lhasa, and attributed to this formation.

Radiometric measurements previously obtained, mainly by using the K-Ar method on biotite^{5,6}, gave ages from 120 to 70 Myr for the dioritic-granodioritic Kangdese plutons. To get further data on the age of these rocks, we have collected several samples for ³⁹Ar/⁴⁰Ar dating, both in the upper andesitic series (Lingzizong), and in the dioritic plutons of the southern part of the Kangdese belt (Fig. 1). The samples that we consider here are: a biotite of a grey porphyritic andesite (XT 59) from the pass north of Lingzhu (91°16'–30°07'); and a biotite and hornblende of a hypersthene-bearing diorite, with large

poecilitic mica (XT 144–145), 5 km north of Taquka (89°36'–29°23').

The ³⁹Ar/⁴⁰Ar stepwise heating method has been applied to pure mineral fractions of biotites and amphiboles, the analytical procedure has been described elsewhere⁷. All the fractions have been irradiated together for 24 h in the core of the high flux reactor of the CENG (Grenoble, France). The results of the argon analysis are given in Table 1. Corrections for atmospheric and neutron induced masses have been calculated—all isotopic masses are corrected from blank of the furnace and line for every step.

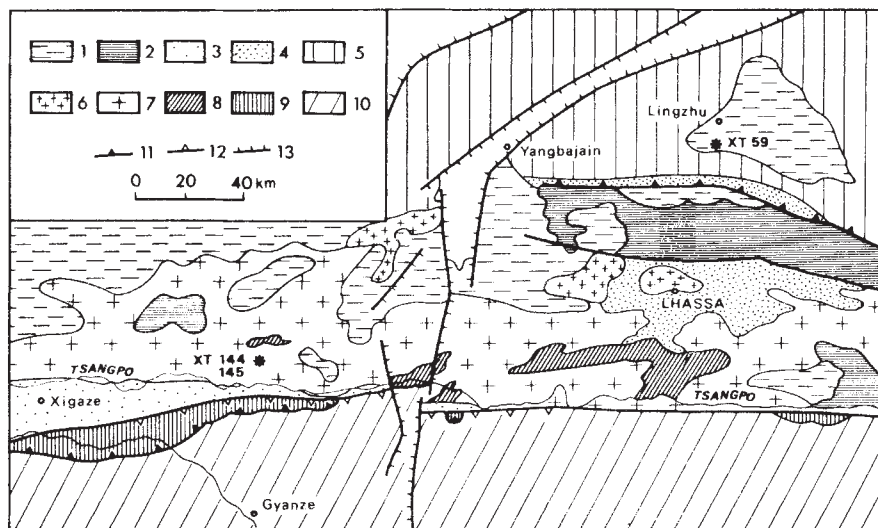
Figures 2 and 3 show age data plotted against cumulative ³⁹Ar. The age spectrum obtained on the biotites extracted from

Table 1 Results of the argon analysis

T(°C)	⁴⁰ Ar*/ ³⁹ Ar	³⁷ Ar/ ³⁹ Ar	³⁶ Ar/ ³⁹ Ar (×10 ⁻³)	³⁹ Ar (%)	Age (Myr)
XT 59 biotite					
500	3.41	0.223	6.93	0.39	83.7 ± 3.2
635	1.95	0.140	7.80	2.65	48.4 2.8
695	2.41	0.031	5.40	4.47	59.6 2.3
755	2.43	0.016	1.53	16.65	60.1 1.5
810	2.41	0.010	1.06	22.47	59.6 1.4
870	2.40	0.008	0.88	14.42	59.5 1.4
920	2.42	0.018	1.44	8.26	59.9 1.6
970	2.42	0.058	2.45	4.97	59.9 1.7
1,070	2.46	0.111	2.20	4.35	60.9 1.7
1,170	2.33	0.150	2.06	6.14	57.7 1.6
1,240	2.41	0.050	1.60	8.51	59.6 2.0
1,300	2.43	0.001	1.96	6.62	60.1 1.7
Fusion	3.56	—	16.53	0.06	87.4 5.5
XT 144 biotite					
350	6.82	—	104.4	0.01	163.8 ± 27.7
500	4.63	0.120	19.95	0.25	112.9 6.7
635	4.62	0.125	5.69	3.01	112.6 3.4
695	4.52	0.009	1.16	12.31	110.2 2.6
755	4.55	0.007	0.03	17.82	110.8 2.4
810	4.57	0.010	0.25	11.20	111.4 2.5
870	4.56	0.019	0.82	6.10	111.1 2.5
920	4.56	0.046	1.97	3.85	111.1 2.7
1,010	4.56	0.137	0.80	7.28	111.1 2.6
1,090	4.51	0.235	0.44	18.10	110.0 2.4
1,200	4.56	0.125	0.44	18.84	111.1 2.5
Fusion	4.52	5.778	2.97	1.20	110.3 2.8
XT 144 amphibole					
450	7.54	2.300	164.24	0.09	180.2 ± 40.6
600	6.19	2.080	267.24	0.16	149.1 65.8
695	4.63	0.963	59.72	0.83	112.9 16.4
760	4.55	0.729	15.81	2.01	111.0 6.0
870	4.65	1.694	26.44	1.29	113.3 11.0
970	4.77	3.900	18.30	5.27	116.0 6.0
1,070	4.59	4.798	3.42	54.02	111.9 3.0
1,170	4.64	4.819	1.61	34.72	113.2 2.7
Fusion	5.25	40.32	21.40	1.58	127.3 5.0
XT 145 biotite					
		× 10 ⁻³			
350	5.46	—	14.40	0.06	132.3 ± 38
500	3.86	67.2	48.42	0.30	94.4 14
635	3.56	6.9	0.47	11.37	87.3 2.7
695	3.76	3.9	0.46	25.51	92.2 2.1
755	3.77	3.4	0.32	26.97	92.3 2.0
810	3.84	4.6	0.26	9.01	94.2 2.1
870	3.78	9.9	0.92	3.37	92.7 2.1
970	3.81	25.0	0.51	9.15	93.3 2.1
1,070	3.78	178.4	0.46	11.21	92.7 2.1
1,090	3.96	496.3	—	2.58	96.9 2.1
1,170	4.07	659.6	0.17	0.42	99.6 2.2
Fusion	18.60	25,979.5	308.3	0.03	416.4 47.0
XT 145 amphibole					
500	5.25	0.76	97.1	0.42	127.3 ± 16.3
635	4.46	0.72	44.0	0.34	108.8 12.6
755	4.23	0.70	14.0	1.20	103.3 5.2
870	4.22	2.16	19.0	0.96	103.1 6.2
970	5.02	4.61	6.5	9.52	122.0 3.7
1,070	4.25	5.19	2.8	62.56	103.8 2.6
1,170	4.12	7.24	1.5	23.40	100.8 2.5
Fusion	4.29	12.00	7.7	1.60	104.7 3.4

Isotopic ratios are corrected from the induced masses interferences. ⁴⁰Ar* refers to radiogenic argon. The monitor sample was the hornblende MMHB for which ⁴⁰Ar*/³⁹Ar = 23.99 after irradiation for 24 h (CENG, Grenoble, France).

Fig. 1 1, Upper Cretaceous–Palaeocene (andesites and ignimbrites); 2, Middle–Upper Cretaceous (Volcano Sedimentary series); 3, Xigaze series; 4, Mesozoic sediments of the Lhasa block (Triassic to Lower Cretaceous); 5, Palaeozoic Precambrian; 6, later granitic bodies; 7, trans-Himalayan batholith; 8, amphibolites; 9, Tsang Po ophiolitic series; 10, Mesozoic Himalayan sediments; 11, main thrust faults; 12, later reverse faults; 13, normal faults.



the andesite formation (XT 59) gives homogeneous apparent ages for intermediate and high-temperature steps. An age plateau can be calculated with the average value of 59.3 ± 2 Myr. This plateau is slightly disturbed only for the first two steps which give 83 ± 3 and 48 ± 3 Myr respectively. As these ages are only related to a weak degassing of ^{40}Ar and a high atmospheric argon content, the evidence for a late disturbing event which could have affected the less retentive sites is not obvious. As for the other samples no clear diffusion age can be deduced from these low temperature ages. Moreover field observations, such as the very weak tectonic deformation of this upper series and very scarce cross-cutting intrusives, support this conclusion. On the other hand, there is no reason for suspecting any excess argon present in the different sites of this biotite. These data show that the age of 59.3 ± 2 Myr can be retained for the emplacement of this andesite formation. This age agrees well with field observations which show the andesitic series overlying unconformably a folded Middle–Upper Cretaceous red bed series (Takena).

Biotites and amphiboles have been extracted from two samples of the plutonic part of the trans-Himalayan belt (XT 144–145). The age spectra for the biotite and the amphibole XT 144 have the same trend: plateau age are well defined with an average value of 111 ± 2 and 113 ± 2 Myr respectively. For the biotites, only the first step displays an older age of 164 ± 60 Myr. In fact, this step is related to 0.02 and 0.01% of ^{40}Ar and ^{39}Ar respectively; moreover, the atmospheric correction, for which the accurate measurement of ^{36}Ar is critical, is mainly responsible for higher error values. The high amount of Ca-derived ^{37}Ar is probably due to observable micro-inclusions of sphene inside the biotites. These sphenes have not been extracted, so that a reasonable and homogeneous granulometry for all the mineral samples (125 μm) can be maintained. The behaviour of the degassing at low temperatures is the same for the amphibole as for the biotite: discordant old ages are yielded by the first two steps, (180 ± 40 and 150 ± 65 Myr), and by the melting step which gives an age of 127 ± 5 Myr. The trend of

these two spectra is typical for undisturbed samples which have not suffered significant loss of radiogenic argon after they have been emplaced. As the amphibole and the biotite yield the same age, we can assume that the emplacement of the intrusion occurred between 111 and 113 Myr ago.

The samples XT 145 is from the same area and has similarities to XT 144 except that the biotite is even more poecilitic. The age plateau of the biotite is also well defined with an average of 93 ± 2 Myr. As for the XT 144 biotite, the low temperature steps yielded older ages than the average, with values of 132 ± 38 Myr. The extremely high value given by the fusion step (416.4 ± 47 Myr), which is inconsistent with the plateau age, cannot be retained for any interpretation and seems more related to the very low value of 0.03% of ^{39}Ar degassed, which is in the lower limit of our measurements.

The age spectrum of the XT 145 amphibole yields a plateau age of 104 ± 2 Myr. This value is obtained if the apparent age of the 970°C step is disregarded for which an inconsistent age of 122 ± 4 Myr has been obtained. The age of 104 ± 2 Myr for

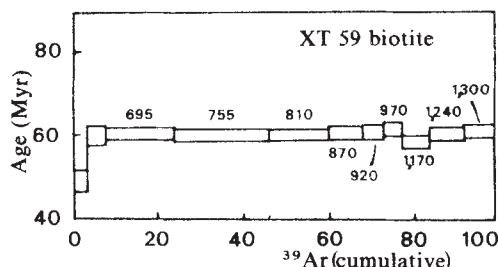


Fig. 2 Age spectra of the biotite of the andesitic formation of Lingzhu.

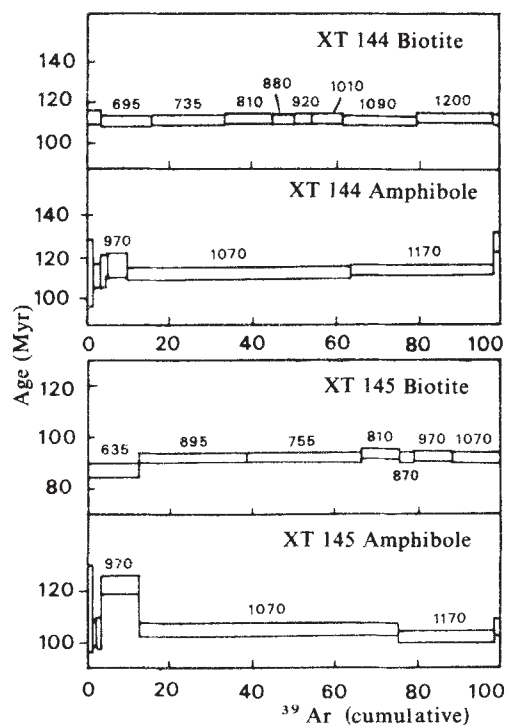


Fig. 3 Age spectra of the biotites and amphiboles of the trans-Himalayan plutonic belt.

this amphibole is significantly higher than that yielded by the biotite of the same sample. As no disturbing effect can be either clearly assumed for this sample one can suppose that the biotite has suffered a continuous argon loss, maybe during cooling, until its late closure at 93 Myr. This explanation implies that the age of 104 Myr yielded by the amphibole would represent either the age of the intrusion or a minimum age value. The better retentivity of the amphibole is well known and supports this view.

The comparison with the age of XT 144 (111–113 Myr) shows that the gabbro diorite formation is in fact made up of a series of intrusions which have occurred at least between 113 and 104 Myr. On the other hand, the occurrence of excess argon trapped in the XT 145 amphibole cannot be totally discarded. As we have outlined above, the apparent age of 120 Myr yielded by the step at 970 °C could be evidence of some radiogenic

excess argon inhomogeneously distributed among the sites of argon retention. This point has to be verified with more analyses on this formation, especially using other radiometric methods.

The ages obtained on these two sampling localities agree quite well with field observations and previous radiometric dating.

Note that consistent ages have been obtained on the 'Ladakh pluton', which is supposed to be a western equivalent of the Kangdese belt, north of the Indus Suture: 120–60 Myr by Rb/Sr whole rock isochrons (A. Gansser, personal communication) and 82 ± 6 Myr by $^{39}\text{Ar}/^{40}\text{Ar}$ method on hornblende⁸.

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Pathway and distribution of trace elements in Lake Vanda, Antarctica

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Lake Vanda, an Antarctic saline lake, situated in the Dry Valleys area, Victoria Land, has a unique distribution of temperature, chlorinity and nutrient matters. Although the origins of these properties and major elements have been discussed previously^{1–6}, only a few geochemical studies have been undertaken on trace elements^{7,8}. We report here concentrations of 14 trace elements, including 7 elements not previously measured (Sc, Cr, Co, Rb, Sb, Cs and Th), in the lake that were determined by instrumental neutron activation and atomic absorption methods. The concentration of every element except for aluminium increased abruptly in the highly stratified deep layer. The chemical composition of inflow water was the same as those of tropospheric aerosol particles^{9,10} and snow^{11,12} at Antarctica. We suggest that the pathway of the trace elements observed in Lake Vanda was: tropospheric aerosol particles → precipitation → glacier → glacial melt water → Lake Vanda. The composition of these elements in the highly stratified layer has been secondary transformed by the reduction, removal and ion-exchange processes.

Lake Vanda (77°32'S, 161°32'E), situated in Wright Valley, Victoria Land, Antarctica, is covered with 3-m thick ice perennially. During the austral summer season, the lake is supplied with glacial melt water mainly from the Onyx River. The lake has no outflow so water is lost only by sublimation of the surface ice.

Chlorinity and temperature increase abruptly from 45 m depth to the bottom ($Cl_{\max} = 74.28$ g per kg, $T_{\max} = 24.3$ °C) (ref. 5) because the layer is highly stratified. Boswell *et al.*^{7,8} reported on the concentrations of six trace elements in the bottom water; we now outline the vertical distributions of Al, Sc, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Sb, Cs and Th and discuss the pathway of these trace elements to Lake Vanda.

Samples were taken in the austral summer season (7–9 January 1979) at 10 depths and at the inflow (Onyx River). The bottom of the sampling site was 64.8-m deep. The con-

centrations of Al, Fe, Ni and Cu were determined by the carbon furnace atomic absorption spectrometry after solvent extraction¹³ and the other elements were determined by the instrumental neutron activation analysis.

The results on 14 elements are presented in Table 1. Every element except aluminium tends to increase with depth and the

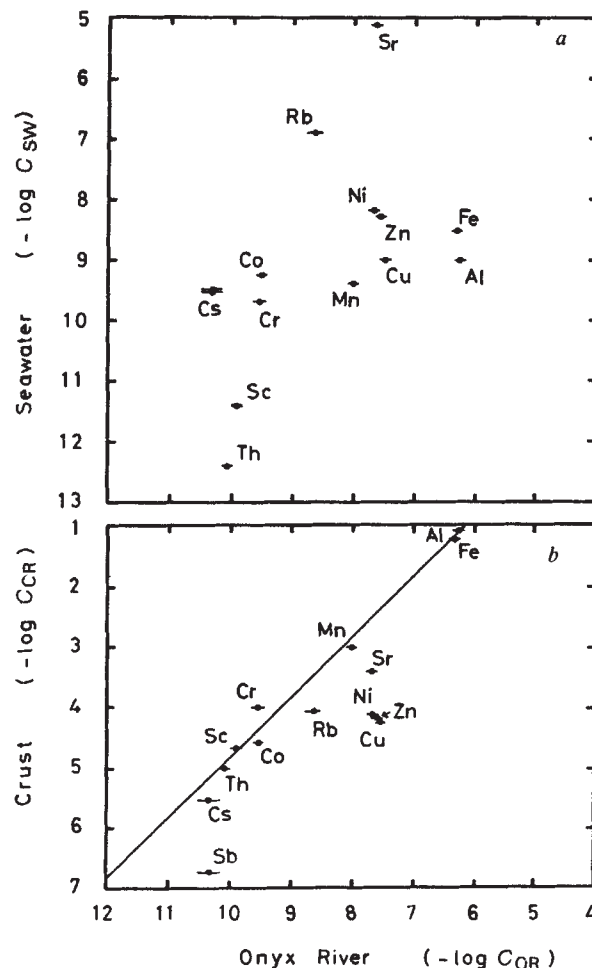


Fig. 1 a, Relationship of trace element contents between Onyx River and seawater. C_{sw} indicates the concentration of seawater after ref. 23 and C_{OR} indicates the concentration of Onyx River (inflow of Lake Vanda) in g per g water. b, Relationship of trace element contents between Onyx River and the continental crust. C_{CR} indicates the concentration of the continental crust after ref. 24 in g per g crust.

Table 1 Analytical results on trace elements of Onyx River and Lake Vanda, Victoria Land, Antarctica ($\mu\text{g per kg H}_2\text{O}$)

Sample	Al*	Sc	Cr	Mn†	Fe*	Co	Ni*
Onyx River	540 $\pm$ 30	0.13 $\pm$ 0.01	0.82 $\pm$ 0.08	10 $\pm$ 1	500 $\pm$ 30	0.30 $\pm$ 0.02	23.2 $\pm$ 1.0
Lake Vanda (depth m)							
4	6.4 $\pm$ 0.5	0.05 $\pm$ 0.02	0.7 $\pm$ 0.2	2.9 $\pm$ 0.3	16.4 $\pm$ 0.8	0.30 $\pm$ 0.02	20.0 $\pm$ 1.0
10	7.8 $\pm$ 0.5	0.0026 $\pm$ 0.0006	0.33 $\pm$ 0.09	2.7 $\pm$ 0.3	15.6 $\pm$ 0.8	0.13 $\pm$ 0.02	8.9 $\pm$ 0.5
20	9.8 $\pm$ 0.5	0.003 $\pm$ 0.002	<0.3	4.4 $\pm$ 0.3	19.2 $\pm$ 0.8	0.12 $\pm$ 0.04	8.9 $\pm$ 0.5
30	6.0 $\pm$ 0.5	<0.002	<0.3	2.5 $\pm$ 0.3	11.2 $\pm$ 0.8	0.13 $\pm$ 0.03	6.7 $\pm$ 0.5
40	8.1 $\pm$ 0.5	<0.002	0.5 $\pm$ 0.3	2.4 $\pm$ 0.3	18.0 $\pm$ 0.8	0.16 $\pm$ 0.03	6.7 $\pm$ 0.5
45	9.9 $\pm$ 0.5	0.002 $\pm$ 0.002	0.30 $\pm$ 0.08	34 $\pm$ 1	32.0 $\pm$ 2.0	0.33 $\pm$ 0.02	13.3 $\pm$ 0.7
50	11.2 $\pm$ 0.5	0.006 $\pm$ 0.002	0.6 $\pm$ 0.5	19 $\pm$ 1	22.0 $\pm$ 2.0	0.12 $\pm$ 0.02	11.1 $\pm$ 0.7
55	8.1 $\pm$ 0.5	0.018 $\pm$ 0.009	<2	350 $\pm$ 10	14.0 $\pm$ 0.8	0.68 $\pm$ 0.09	37.7 $\pm$ 2.0
60	12.7 $\pm$ 0.5	0.34 $\pm$ 0.02	<4	2,100 $\pm$ 100	180 $\pm$ 10	2.0 $\pm$ 0.2	70 $\pm$ 4
64	8.1 $\pm$ 0.5	0.19 $\pm$ 0.03	<6	2,900 $\pm$ 100	500 $\pm$ 30	1.7 $\pm$ 0.3	175 $\pm$ 10
Sample	Zn	Cu*	Rb	Sr	Sb	Cs	Th
Onyx River	28 $\pm$ 4*	31.3 $\pm$ 1.5	2.3 $\pm$ 0.6	22 $\pm$ 4	0.05 $\pm$ 0.02	0.05 $\pm$ 0.02	0.090 $\pm$ 0.008
Lake Vanda (depth m)							
4	39 $\pm$ 3	37.4 $\pm$ 1.5	3.0 $\pm$ 0.7	280 $\pm$ 20	0.07 $\pm$ 0.03	0.04 $\pm$ 0.02	<0.01
10	14 $\pm$ 1	10.6 $\pm$ 0.5	3.2 $\pm$ 0.8	210 $\pm$ 20	0.03 $\pm$ 0.02	0.007 $\pm$ 0.006	0.016 $\pm$ 0.008
20	23 $\pm$ 3	16.9 $\pm$ 1.0	4 $\pm$ 2	340 $\pm$ 30	<0.05	<0.03	0.03 $\pm$ 0.03
30	44 $\pm$ 4	24.4 $\pm$ 1.5	4 $\pm$ 2	540 $\pm$ 30	<0.05	<0.03	<0.03
40	36 $\pm$ 3	29.1 $\pm$ 1.5	6 $\pm$ 3	590 $\pm$ 30	0.14 $\pm$ 0.05	<0.02	<0.03
45	60 $\pm$ 4	30.1 $\pm$ 1.5	8 $\pm$ 2	970 $\pm$ 30	0.05 $\pm$ 0.03	0.04 $\pm$ 0.02	<0.01
50	100 $\pm$ 10	75 $\pm$ 5	15 $\pm$ 3	2,800 $\pm$ 200	0.05 $\pm$ 0.02	0.03 $\pm$ 0.02	0.04 $\pm$ 0.004
55	320 $\pm$ 20	650 $\pm$ 30	60 $\pm$ 20	18,000 $\pm$ 1,000	0.22 $\pm$ 0.09	<0.1	<0.2
60	480 $\pm$ 20	970 $\pm$ 50	160 $\pm$ 40	36,000 $\pm$ 2,000	10 $\pm$ 2	1.2 $\pm$ 0.2	1.7 $\pm$ 0.4
64	700 $\pm$ 30	830 $\pm$ 50	190 $\pm$ 40	60,000 $\pm$ 3,000	3.1 $\pm$ 0.5	1.3 $\pm$ 0.3	1.0 $\pm$ 0.5

All the lake water samples were taken with a 1-l Kitahara-type sampler made of stainless steel, and the Onyx River sample was taken with a non-metal plastic jug. The polyethylene bottles used were previously soaked in 6 M nitric acid for 1 week. Super special grade hydrochloric acid was added to the samples to store at pH 1.5–1.8, then the bottles were wrapped in polyethylene bags. Results were obtained by instrumental neutron activation analysis with TRIGA II type reactor (Rikkyo University, thermal neutron flux; $5 \times 10^{11} \text{ n cm}^{-2} \text{ s}^{-1}$) and Ge(Li) detector (EG&G ORTEC Corp., effective volume 25cc), 4096 ch M.C.A. (Northern Corp.). Unfiltered 100-ml samples were dried up and the residue was irradiated for 18 h, cooled for 1 month and counted for 3–18 h. The error denotes the analytical standard deviation of the uncertainties due to counting statistics. The AGV-1 (ref. 25) (USGS) was used for the standard of Sc, Rb, Sr, Sb, Cs and Th.

* Results of Al, Fe, Ni, Cu and Zn (Onyx River only) were obtained by carbon furnace atomic absorption spectrometry. The error denotes the analytical standard deviation.

† Results of Mn were obtained by instrumental neutron activation analysis (with F-ring, $n = 1.0 \times 10^{12} \text{ n cm}^{-2} \text{ s}^{-1}$). The water from 0.5 to 10 ml was subsampled to quartz tubes, then irradiated for 10 min and cooled 30 min. Manganese carrier (Mn^{2+} 0.2 mg ml $^{-1}$) was added to the irradiated samples and then the precipitate of manganese dioxide was made. After filtration, the precipitate was counted for 5–40 min. The chemical yield was determined by reirradiation for 1–2 s and recounted for 30 s after 1 h cooling.

profiles resemble those of chlorinity and temperature⁵. The concentration of aluminium was nearly constant at all depths. Wilson¹⁴ suggested that a climatic change occurred ~1,200 yr ago and that then glacial melt water flowed on top of the nearly evaporated palaeolake water, since when the calcium chloride of the palaeolake water has diffused upward. The same diffusion process was considered to have produced the observed profiles for all of the trace elements except aluminium. Aluminium would not be dissolved even in the reducing conditions of the Lake Vanda deep water, and could not be diffused upwards.

The main purpose of the geochemical study on Lake Vanda was to elucidate the origin and pathway of the highly concentrated salts. Many reports^{4,6,15–17} have discussed the origin of major elements. There exist, however, few geochemical studies on trace elements^{7,8} in Lake Vanda. Four possible origins of trace elements were considered: (1) connate seawater; (2) dissolution by rock weathering; (3) grounded marine ice shelf; and (4) aerosol particles. The Dry Valley Drilling Project (DVDP) studies provide evidence that in Miocene period there was a fjord in Wright Valley¹⁸. Nakai *et al.*⁶ concluded from isotope studies that the sulphate in the lake was derived directly from the ocean. Results obtained for trace elements do not support this conclusion because the chemical composition of the inflow water (Fig. 1a) and the 64-m layer are not identical with those of seawater. Connate seawater and oceanic seasalt could not play a major part in the origin of trace elements in Lake Vanda. Considerable rock weathering materials have been brought to the lake through the Onyx River (43.4 tonnes from Onyx River, 1974–75 summer season¹⁹). The relationship of the trace element content between Onyx River and the continental crust is shown in Fig. 1b. Assuming that aluminium was a constituent element of the crust, materials of crustal origin were a major source for Fe, Mn, Co, Sc, Th and Cs and a minor source for Sr, Rb, Ni, Cu and Zn.

Denton *et al.*²⁰ suggested that a part of the Ross Ice Shelf moved into the mouth of the Wright Valley during the last Wisconsin (Würm) glacial period. Onyx River is a meltwater stream mainly from the Lower Wright Glacier which situated in the mouth of the Wright Valley. If this marine ice sheet in McMurdo Sound had been grounded and had moved into the mouth of the valley, then the water of Onyx River might reflect the marine source of the ice body. Matsubaya *et al.*²¹, however, reported the same isotopic ratios of δD and $\delta^{18}\text{O}$ of Onyx River as those of alpine glaciers in the coastal region of the Dry Valleys. Therefore, the present day meltwater does not seem to come directly from grounded marine ice shelf.

The relationship of the trace element contents between Onyx River and tropospheric aerosol particles at the South Pole⁹ is shown in Fig. 2a. The chemical compositions of inflow water and aerosol particles show a good agreement except for Sb, and they also have a similar composition to Antarctic snow^{11,12}. The aerosol particles over the Antarctic and remote oceanic²² regions are less enriched in Fe, Mn, Co, Sc, Th and Cs compared with the crustal abundance (Al as a constituent element) and this would not conflict with the explanation of aerosol particles origin of trace elements in Lake Vanda. Elements, such as Cu, Ni and Zn, in Onyx River that are anomalously enriched compared with seawater and the crust are also enriched in the case for aerosol particles in the Antarctic region^{9,10}.

The results therefore support the conclusion that the pathway of the trace elements observed was: tropospheric aerosol particles $\rightarrow$ precipitation $\rightarrow$ glacier $\rightarrow$ glacial melt water $\rightarrow$ Lake Vanda. Connate seawater introduced during the fjord age, seasalt particles, crustal weathering materials and grounded marine ice shelf could not have a major effect on the origin of trace elements in Lake Vanda.

The layer below 45 m deep was produced by a diffusion process of highly concentrated palaeolake water. The 60–64 m

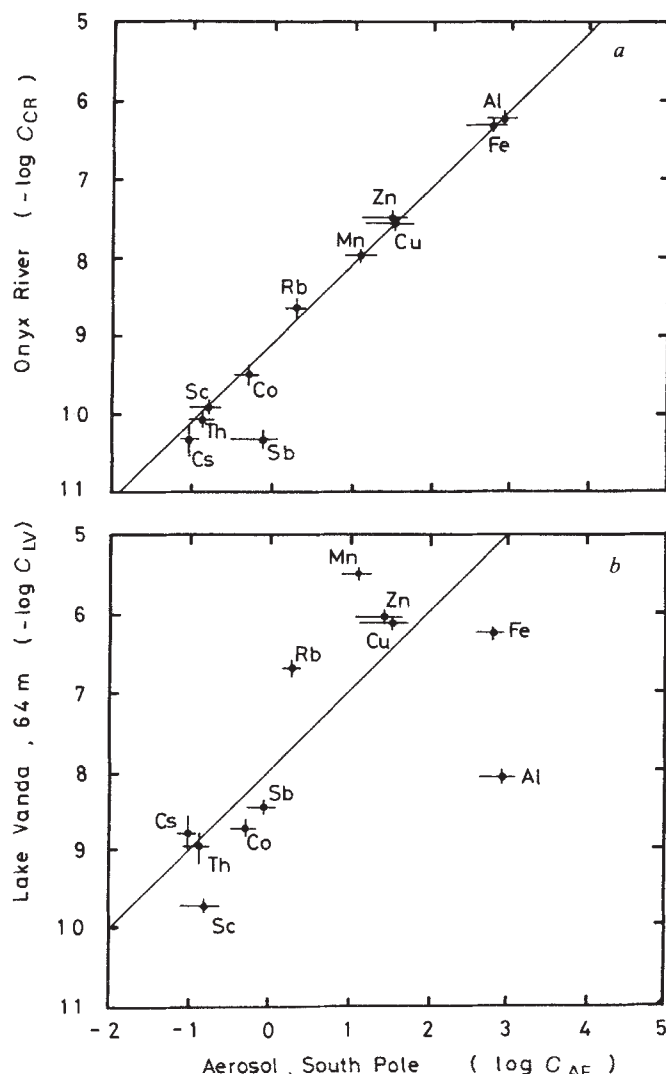


Fig. 2 a, Relationship of trace element contents between Onyx River and the aerosol particles at South Pole. C_{AE} indicates the concentration of trace element contents in aerosol at South Pole¹⁰ in pg m^{-3} STP. b, Relationship of trace element contents between Lake Vanda, 64 m and South Pole aerosol. C_{LV} indicates the trace element contents of Lake Vanda, 64 m, in g per g water.

layer is remarkably depleted in dissolved oxygen and contained hydrogen sulphide⁵. The bottom layer was such that the chemical species in the layer should have a reduced form. The relationship of the trace element contents between Lake Vanda bottom water and aerosol particles at South Pole is shown in Fig. 2b. The elements, Cs, Th, Co, Sb, Cu and Zn still have a good correlation with aerosol particles. Manganese would have been reduced in the bottom sediment and diffused upwards to produce an anomalously high concentration in deep water. Both aluminium and scandium precipitate and are not dissolved in the reducing condition of the deep water. Thus, aluminium and scandium give the relatively low concentrations observed. The concentration of iron is nearly constant in the upper layers above 55 m, but increases with depth in the layer 60–64 m where iron is reduced to a soluble form, subsequently diffused upwards and then precipitates. The enrichment of certain elements such as Zn and Cu in the bottom water would also be due to processes taking place at the sediment–water interface.

The correlation between aerosol particles and the constituents, except for Mn, Al, Sc, and Fe mentioned above, indicates that the trace elements in the deep water might also be derived from aerosol particles although the chemical compositions were somewhat different from each other.

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Soil carbon pools and world life zones

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Soil organic carbon in active exchange with the atmosphere constitutes approximately two-thirds of the carbon in terrestrial ecosystems^{1,2}. The relatively large size and long residence time of this pool (of the order of 1,200 yr) make it a potentially important sink for carbon released to the atmosphere by fossil fuel combustion; however, in many cases, human disturbance has caused a decrease in soil carbon storage^{3,4}. Various recent estimates place the global total of soil carbon between 700 (ref. 2) and $2,946 \times 10^{15}$ g (ref. 5) with several intermediate estimates: 1,080 (ref. 1), 1,392 (ref. 6), 1,456 (ref. 3), and $2,070 \times 10^{15}$ g (ref. 7). Schlesinger's³ estimate seems to be based on the most extensive data base (~200 observations, some of which are mean values derived from large studies in particular areas) and is widely cited in carbon cycle studies. In addition to estimating the world soil carbon pool, it is important to establish the relationships between the geographical distribution of soil carbon and climate, vegetation, human development and other factors as a basis for assessing the influence of changes in any of these factors on the global carbon cycle. Our analysis of 2,700 soil profiles, organized on a climate basis using the Holdridge life-zone classification system⁸, indicates relationships between soil carbon density and climate, a major soil forming factor. Soil carbon density generally increases with increasing precipitation, and there is an increase in soil carbon with decreasing temperature for any particular level of precipitation. When the potential evapotranspiration equals annual precipitation, soil carbon density⁹ is $\sim 10 \text{ kg m}^{-2}$, exceptions to this being warm temperate and subtropical soils. Based on recent estimates of the areal extent of major ecosystem complexes^{9,10} which correspond well with climatic life zones, the global soil organic carbon pool is estimated to be $\sim 1,395 \times 10^{15}$ g.

The amount of carbon in soil is the balance between inputs of organic material from the biota, which ultimately depend on the type of vegetation and its productivity at a particular site, and losses primarily through heterotrophic respiration. In turn, these fluxes and their balance depend on climatic conditions such as temperature and moisture. As a result, soil carbon density is strongly correlated with climatic variables, and the general world-scale distribution of soil carbon density should be closely related to climatic patterns⁴.

Several data sets demonstrating relationships between soil organic matter and climatic indices have been documented by Jenny^{4,11}. Kononova¹² has described an analysis of humus content of soils of the USSR by Volobuev¹³ which shows a pronounced relationship between this soil characteristic and a climate index combining temperature and precipitation.

The present analysis is based on 2,696 soil profiles representing virtually every major life zone. Samples from each soil horizon or from standard depth increments (0, 5, 10, 20, 40, 60, 80, 100 cm) were analysed for carbon content. Carbon in the litter layer was not included in these profiles. Bulk density samples were obtained for each layer using a tube of known volume.

Profiles were obtained from the literature to augment the number of life zones represented. Where soil bulk density was not reported, it was estimated from carbon content and depth using the regression

$$B_D = b_0 + b_1 D + b_2 \log_{10} c_t$$

where B_D is bulk density, D is depth to centre of horizon or layer and c_t is the fraction by mass of organic carbon^{14,15}. This relationship was calibrated against data for which the bulk density was known for a wide range of vegetation types. Bulk density does not vary greatly between profiles from the same vegetation type and therefore does not present serious difficulties for profile analysis.

The density of carbon in a horizon (or other depth increment) of unit area (1 m²) was calculated as

$$c = c_t B_D (1 - \delta_{2mm}) V$$

where c is carbon density, δ_{2mm} is the fraction of material larger than 2 mm diameter and V is the volume of the horizon or layer. Data for the soil layers have then been summarized to indicate soil carbon density on a square metre basis to 1 m depth. (The complete data set and documentation are available from P.J.Z.)

Soil profiles were selected with the intention of sampling ecosystems that are not disturbed to the point of obscuring basic relationships between carbon storage and climate. However, temperate forest soil samples are often from second growth stands or forests that are established on abandoned fields, and soils in some life zones, such as steppe and thorn steppe, have almost always been disturbed by human management or grazing by domestic animals. Soils from wetlands and cultivated lands were not included in this data set.

The data were organized according to the Holdridge Life Zone Classification System^{8,16} which assigns a life zone with which vegetation complexes can be associated based on the climate variables, biotemperature and annual precipitation (Fig. 1). Biotemperature is defined as the annual mean of unit-period temperatures with substitution of zero for all temperature values below 0 °C and above 30 °C. The Holdridge life-zone approach to classifying vegetation has been empirically tested with good results¹⁷⁻¹⁹. Based on field data and experience, vegetation can indicate the appropriate life-zone classification in the absence of climate data. This was used to classify soil profiles for which adequate climatic information for direct classification was not available. The life-zone summary of soil carbon data is given in Table 1, and contours of soil carbon density based on this are plotted in Fig. 1.

Most of the soil profiles are from forest life zones, particularly in the tropical and cool temperate regions. Fewer samples are from particularly wet-life zones of lower latitudinal or

altitudinal regions. These extremely wet regions occupy little of the Earth's surface, but they could contain appreciable pools of soil carbon due to high soil carbon density. Other life zones of uncertain significance inadequately represented are cold deserts (dry tundra and boreal desert) that occupy a small amount of land surface area. Warm and hot desert life zones are also poorly sampled but their carbon contents are low.

The frequency distribution of soil carbon density for each life zone is generally skewed due to a few profiles with relatively high carbon content. These high carbon density profiles correspond to local site conditions (for example, topography or microclimate) which are not typical of the life zone as a whole; however, such soil profiles are found in any reasonable sample. A maximum likelihood fit of the three-parameter Weibull distribution²⁰, whose probability density function is

$$f(t, \eta, \sigma, \mu) = \begin{cases} \frac{\eta}{\sigma} \left(\frac{t-\mu}{\sigma} \right)^{\eta-1} \exp \left[- \left(\frac{t-\mu}{\sigma} \right)^{\eta} \right] & t \geq \mu \\ 0 & t < \mu \end{cases}$$

$\eta, \sigma > 0$

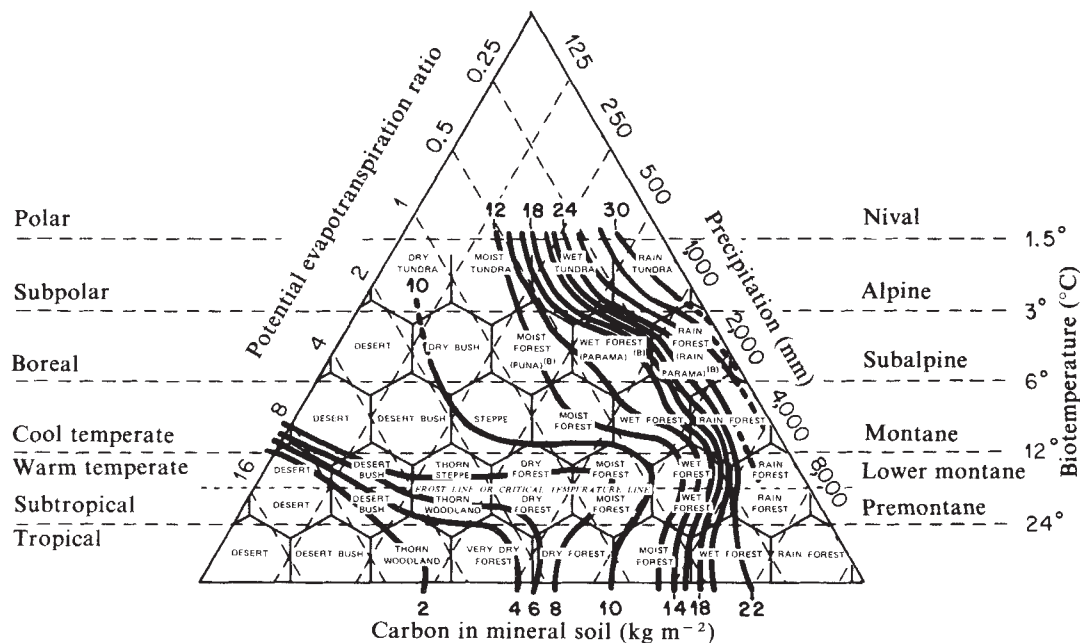
conveniently summarizes the character of the skewed distribution of soil carbon density in each life zone. This distribution can have a variety of shapes; fitting it to soil carbon density resulted in single-peaked curves with modes as recorded in Table 1. The degree of skewness is indicated by the difference between the mean and mode.

The size of the world organic soil carbon pool is estimated by associating the life-zone classification of soil profiles (Table 1) with estimates of the area of major ecosystem complexes, relying on the correspondence of life zones with vegetation associations^{8,16}. Areas of groups of life zones are indicated in Table 2 based on the recent mapping of major ecosystem complexes by Olson^{9,10}. Soil carbon storage is calculated for each life-zone group using the appropriate mean soil carbon

Table 1 Life-zone summary of soil carbon density data

Life zone	No. of samples	Mean soil carbon density (kg m ⁻²)	Median	s.d.	Mode
Moist tundra	12	10.9	9.0	6.4	4.7
Wet tundra	28	22.2	19.5	16.2	7.7
Rain tundra	8	36.6	34.7	12.9	23.2
Boreal dry bush	8	10.2	8.6	4.6	5.7
Boreal moist forest	222	11.6	10.1	8.2	5.6
Boreal wet forest	15	13.1	10.2	7.1	6.9
Boreal rain forest	15	25.6	28.8	12.2	20.9
Cool temperate desert	4	9.7	10.3	0.7	—
Cool temperate desert bush	129	10.0	8.1	6.0	6.4
Cool temperate steppe	374	13.3	11.2	9.5	6.6
Cool temperate moist forest	616	12.1	10.1	8.2	6.9
Cool temperate wet forest	344	13.9	12.6	8.8	9.2
Warm temperate desert	9	1.4	1.2	1.0	0.3
Warm temperate thorn steppe	291	7.6	5.7	6.8	2.5
Warm temperate dry forest	94	7.9	7.1	3.9	5.6
Warm temperate moist forest	63	6.0	5.3	2.6	4.9
Subtropical thorn woodland	18	5.4	5.0	2.2	4.2
Subtropical dry forest	5	2.6	1.3	4.1	—
Tropical very dry forest	94	6.2	5.5	3.9	3.7
Tropical dry forest	173	10.2	7.4	11.2	2.4
Tropical moist forest	162	11.5	8.1	11.7	1.8
Tropical wet forest	12	21.0	19.9	8.8	17.1

Fig. 1 Contours of soil carbon density plotted on Holdridge schematic for world life-zone classification. Values of biotemperature and precipitation uniquely determine a life zone and associated vegetation. Contour lines for mean soil carbon density are drawn by visual interpolation of the data presented in Table 1. Dash lines indicate probable extrapolation of contour lines in climatically extreme life zones from which data were not available.



densities. Values for wetlands (such as mangroves, swamps and bogs) and cultivated land are from ref. 21. The global organic soil carbon pool estimated by this approach is $1,395 \times 10^{15}$ g.

The contours of soil carbon density displayed in Fig. 1 reflect the balance of input and loss fluxes imposed by climate. Soil carbon density increases from left to right reflecting greater organic matter production with higher annual precipitation. This effect is clearest in the tropics where temperature is not limiting productivity. The latter effect is dominant so that soil carbon density increases on Fig. 1 with decreasing biotemperature for any particular annual precipitation. Organic matter production decreases with temperature, but low temperatures also limit soil organic matter decomposition.

The combined influence of temperature and precipitation is clarified by the third axis of the Holdridge diagram (Fig. 1), the ratio of potential evapotranspiration to annual precipitation. Potential evapotranspiration (PET) is the amount of water that would be evapotranspired at a given biotemperature in constantly optimal conditions of soil moisture and plant cover. PET is taken by Holdridge to be 58.93 times biotemperature⁸.

When this ratio is <1.0 , rainfall exceeds potential evapotranspiration and vice versa. All the life zones bordering the unit potential evapotranspiration ratio line have soil carbon densities of around 10 kg m^{-2} , except in the warm temperate and subtropical belts where a strong seasonality⁹ limits production, but whose decomposition conditions are favourable for most of the year. This introduces an area of low soil carbon density in the life zones of this part of the diagram. Perpendicular to this line, soil carbon density uniformly increases as the PET ratio decreases, indicating not only increased vegetative productivity with humidity but possible inhibition of soil organic matter decomposition in waterlogged soils of the superhumid life zones. Conversely, as the PET ratio increases, or conditions become dryer, soil carbon density decreases.

The variation of soil carbon density within life zones is considerable. Even though we excluded from the analysis profiles from hydromorphic soils, the standard deviation for most life zones is high (Table 1). The fact that the standard deviation does not decrease with more profile data indicates that a large proportion of this variation may be due to soil variation, and increased sampling will do little to reduce it. This variation is attributable to factors such as aspect, topography, parent material, age of soil profile in relationship to current climate and vegetation and so on⁴ that do not appear at the life-zone level of organization.

Changes in global climate can be expected to alter total

storage of carbon in soils. For example, current climate models of the effect of increased atmospheric CO_2 predict that higher latitude regions will become substantially warmer and perhaps

Table 2 Estimate of the world soil carbon pool

Life-zone groups	Area* ($\times 10^{12} \text{ m}^2$)	Carbon density (kg m^{-2})	Soil carbon ($\times 10^{15} \text{ g}$)
Tropical forest—wet	4.1	19.1	78.3
Tropical forest—moist	5.3	11.4	60.4
Tropical forest—dry	2.4	9.9	23.8
Tropical forest—very dry	3.6	6.1	22.0
Temperate forest—warm	8.6	7.1	61.1
Temperate forest—cool	3.4	12.7	43.2
Boreal forest—wet	6.9	19.3	133.2
Boreal forest—moist	4.2	11.6	48.7
Tropical woodland and savanna	24.0	5.4	129.6
Temperate thorn steppe	3.9	7.6	29.6
Cool temperate steppe	9.0	13.3	119.7
Tropical desert bush	1.2	2.08	2.4
Warm desert†	14.0	1.4	19.6
Cool desert†	4.2	9.9	41.6
Boreal desert	2.0	10.2	20.4
Tundra	8.8	21.8	191.8
Sub-total for this study			1,025.4
Cultivated land‡	21.2	7.9	167.5
Wetlands‡	2.8	72.3	202.4
Global soil carbon pool			1,395.3

Categories of life-zone groups:

Tropical forest—wet = tropical rain forest; tropical wet forest; subtropical rain forest; subtropical wet forest.

Tropical forest—moist = tropical moist forest; subtropical moist forest.

Tropical forest—dry = tropical dry forest; subtropical dry forest.

Tropical forest—very dry = tropical very dry forest.

Temperate forest—warm = warm temperate rain forest; warm temperate wet forest; warm temperate moist forest; warm temperate dry forest.

Temperate forest—cool = cool temperate rain forest; cool temperate wet forest; cool temperate moist forest.

Boreal forest—wet = boreal rain forest; boreal wet forest.

Boreal forest—moist = boreal moist forest.

Tropical woodland and savanna = tropical thorn woodland; subtropical thorn woodland.

Temperate thorn steppe = temperate thorn steppe.

Cool temperate steppe = cool temperate steppe.

Tropical desert bush = tropical desert bush; subtropical desert bush.

Warm desert = tropical desert; subtropical desert; warm temperate desert bush; warm temperate desert.

Cool desert = cool temperate desert bush; cool temperate desert.

Boreal desert = boreal dry bush; boreal desert.

Tundra = rain tundra; wet tundra; moist tundra; dry tundra.

* After Olson^{9,10}.

† Ratio of warm desert to cool desert after Walter²⁴.

‡ Carbon density for cultivated land and wetland after Schlesinger²¹.

§ Interpolated from Fig. 1.

dryer²². As we see from Fig. 1, relatively small changes in either temperature or precipitation in subpolar regions may have profound effects on the amount of carbon stored in these carbon rich soils.

The estimate of the world soil carbon pool in Table 2, $1,395 \times 10^{15}$ g, corroborates the intermediate values cited above which have been based on substantially fewer samples. The standard deviation of the global pool estimate is $\sim 200 \times 10^{15}$ g, assuming the variability in soil carbon density (Table 1) to be the only source of error.

Comparing the summary in Table 2 with that of Schlesinger³, differences in several intermediate estimates are evident. Schlesinger estimates the soil carbon pool in tropical forests to be 255×10^{15} g compared with 184.5×10^{15} g for the four categories of tropical forest in Table 2. However, our estimate for tropical thorn woodland, 129.6, is over twice as large as Schlesinger's corresponding estimate for tropical savanna. These differences in soil carbon values in the tropics seem to be due to recognition, in more recent surveys of vegetation extent, that areas assigned by Whittaker and Likens²³ (and used by Schlesinger) to tropical forest are, in many instances, in savanna or parkland. Our estimates for temperate forest and boreal forest agree with those of Schlesinger, while soil carbon density for cool temperate steppe is lower than Schlesinger's leading to a smaller pool size in this category. The differences in naming conventions in desert life zones make comparison with Schlesinger's value difficult, but his higher value may be due to inclusion in desert scrub of areas categorized by Olson as desert. Although a steep gradient in soil carbon densities across tundra life zones is apparent in Fig. 1, these are grouped in available areal extent estimates. This may be a significant source of error.

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Ontogeny of the corticoliberin neuroglandular system in rat brain

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Although it has long been known that the hypothalamus contains substances which stimulate the release of adrenocorticotrophic hormone (ACTH)^{1,2}, corticoliberin (CRF) has only recently been identified and synthesized^{3,4}. Here we describe the results of immunocytochemical staining of the neuroglandular system using an antiserum raised against this synthetic peptide. Neurone bodies containing CRF-like immunoreactivity (CLI) were stained in the paraventricular nuclei (PVN) of colchicine-injected rats whereas in normal rats, CLI was detected only in nerve fibres. These CLI-positive processes were abundant in the zona externa of the median eminence and in the pituitary stalk; they terminated close to capillaries of the primary portal plexus. In the rat fetus, CLI-containing fibres were detected only after the 18th day of development. After birth, a transitory disappearance of CLI was followed by its accumulation in fibres and perikarya. Morphological and physiological results corroborate recent biochemical studies^{3,4}; these will have to be taken into account in studies of the phenomena implicating participation of the adrenal cortex.

Antiserum to CRF was raised in two rabbits immunized with a conjugate prepared by reacting (3 h, 4°C) 1 mg CRF (Bachem), 2 mg bovine serum albumin (BSA) and glutaraldehyde (2.1 µmol) in 0.1 M phosphate buffer solution, pH 7.4. Both rabbits were injected subcutaneously every 7 days for 3 weeks with 50 µl of the conjugate (equivalent to 100 µg CRF) emulsified in complete Freund's adjuvant. Weekly blood samples were collected beginning with the third injection. Anti-BSA antibodies were removed by immunoprecipitation.

The specificity of the anti-CRF antiserum was examined by immunocytochemistry: adjacent sections were incubated with the antiserum previously absorbed either with increasing concentrations of CRF (0.05–1.6 mg ml⁻¹ of undiluted serum) or with high concentrations (>1 mg ml⁻¹) of various heterologous neuropeptides (vasopressin, oxytocin, vasotocin, neurophysin I, neurophysin II, substance P, Leu⁵-enkephalin, β-melanotropin, β-endorphin, ACTH_{17–39}, gonadoliberein and somatostatin). Angiotensin I, angiotensin II and angiotensinogen, which contain a small peptide sequence related to a sequence of the CRF molecule³, were added at a concentration of 20 mg ml⁻¹. Pretreatment of the serum with these heterologous peptides did not alter the staining patterns. Conversely, previous addition of CRF to the anti-CRF antiserum induced a progressive decrease in intensity of staining (for concentrations of 0.05–0.4 mg ml⁻¹). Total abolition of staining was obtained only after addition of large amounts of CRF to the antiserum (>0.8 mg ml⁻¹). This observation, and the weak but specific staining obtained with antiserum diluted to 1:8,000 indicate that anti-CRF antibodies are abundant and/or of high affinity in this antiserum.

We compared the topography of CLI neurones with that of other peptide-containing neurones revealed by antisera against β-endorphin⁵, gonadoliberein⁶, somatostatin⁷, oxytocin, vasopressin and neurophysins⁸ which have been used previously. The present study was performed on (1) five brains from normal adult rats (250–300 g); (2) five brains from rats injected intracisternally with 50 µg colchicine 72 h before killing; and (3) 20 rat brains taken at different stages of development (between days 15 and 20 of fetal development, less than 8 h and more than 12 h after birth, and on the 2nd, 4th and

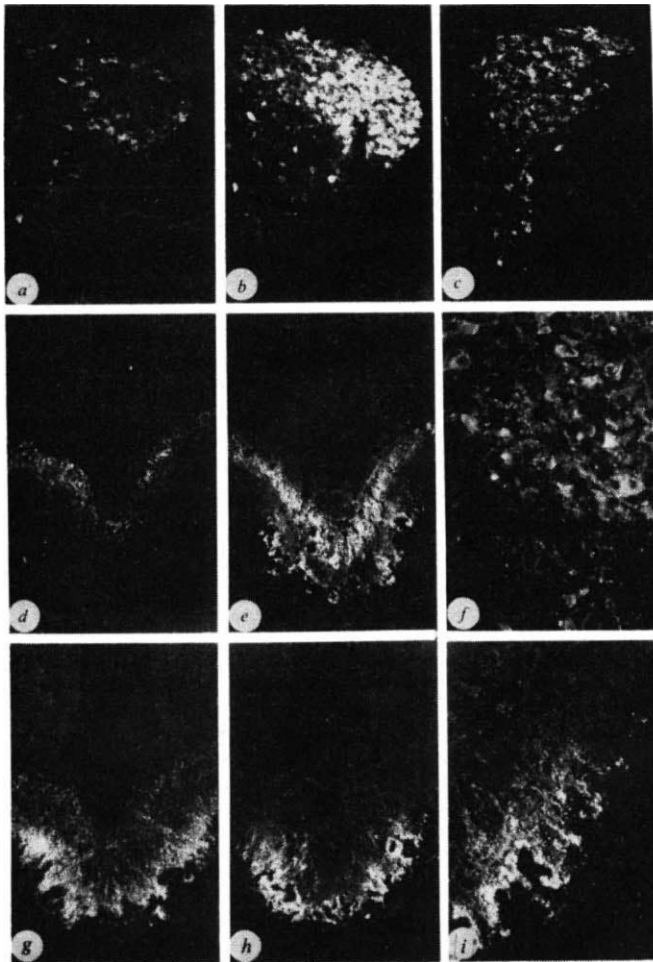


Fig. 1 *a-c*, Adjacent frontal sections showing the different distributions of perikarya stained with antisera against oxytocin (*a*), vasopressin (*b*) and CRF (*c*) in a paraventricular nucleus of a colchicine-treated rat ($\times 50$). *d,e,g,h*, Adjacent frontal sections of the median eminence of an untreated rat, showing the different patterns of immunoreactivity to oxytocin (*d*), vasopressin (*e*), somatostatin (*g*) and CRF (*h*) antisera ($\times 50$). *f,i*, Higher magnification ($\times 260$) of anti-CRF-stained perikarya in the paraventricular nucleus, and CLI-containing perivascular nerve endings in the zona externa of the median eminence.

5th days of their lives). All animals were perfused through the aorta (under chloral anaesthesia in adults) with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Dissected brains or whole fetal heads (before 17 days of development) were postfixed in the same fixative for 3–5 h. After rinsing overnight in sucrose-containing phosphate buffer (adult brains in 10% sucrose, others in 30% sucrose), the brains were frozen in nitrogen vapour at -100°C and cut on a cryostat in serial 10–20 μm -thick sections, which were mounted on gelatinized glass slides. Sections were treated according to the indirect immunofluorescence technique described elsewhere⁵ using antisera diluted 1:100 to 1:400.

In adult untreated rats, the anti-CRF antiserum stained a dense aggregation of nodular processes and endings in all parts of the median eminence (Fig. 1*h, i*). CLI fibres gathered in the anterior and lateral regions of the median eminence, ran in the fibrillar layer of the zona externa where they gave rise to radiating collaterals whose ramifications terminated next to the superficial capillaries of the primary portal plexus, and in the intra-eminential vascular loops (Fig. 1*i*). Note that this pattern of immunoreactivity differs from the distribution of fibres and terminals containing vasopressin, oxytocin, somatostatin, gonadoliberin or β -endorphin (compare *d, e, g, h* in Fig. 1 and *d, e, f, g* in Fig. 2). Colchicine-induced blocking of the axonal flow provoked simultaneously a rarefaction of the CLI-

positive processes and the appearance of numerous immunoreactive perikarya. Anti-CRF-stained perikarya were mainly located within the upper and periventricular parts of the PVN (Fig. 1*c, f*); their distribution differs from that of perikarya stained with anti-oxytocin or anti-vasopressin antisera (Fig. 1*a-c*). Further investigations using double-antibody staining techniques, comparisons of adjacent semi-thin sections processed with different antisera, and ultrastructural analyses, are in progress to determine whether or not all CLI-positive neurones are different from those positive for vasopressin or oxytocin. Some immunoreactive cell bodies could also be seen isolated or grouped in small clusters in the lateral hypothalamus (especially in perifornical areas) and in the mediobasal hypothalamus. CLI perikarya were absent from the supraoptical and suprachiasmatic nuclei.

In fetal hypothalami, a few CLI-positive fibres were detected, beginning with day 18 of their development, in the anterior regions of the median eminence. At day 19 they were more numerous and extended towards the pituitary stalk; they began to establish neuro-humoral connections with the portal capillaries at the periphery of the zona externa (Fig. 2*a*). During the first 8 h after birth, CLI-fibres could no longer be detected in the median eminence; perhaps the stress of birth resulted in neurone depletion of CLI. Four hours later, immunoreactive fibres could be seen again (Fig. 2*e*); moreover, the accumulation of CLI enabled some perikarya to be detected in the PVN (Fig. 2*c*).

The specificity of localization of CLI neurones in adult and fetal brains is supported by the following evidence: (1) the positive CLI response in perikarya and processes was removed by preabsorption of the anti-CRF antiserum with synthetic CRF but not by preabsorption with other neuropeptides, particularly angiotensin I, angiotensin II and angiotensinogen. Thus, anti-CRF antiserum does not stain the previously described

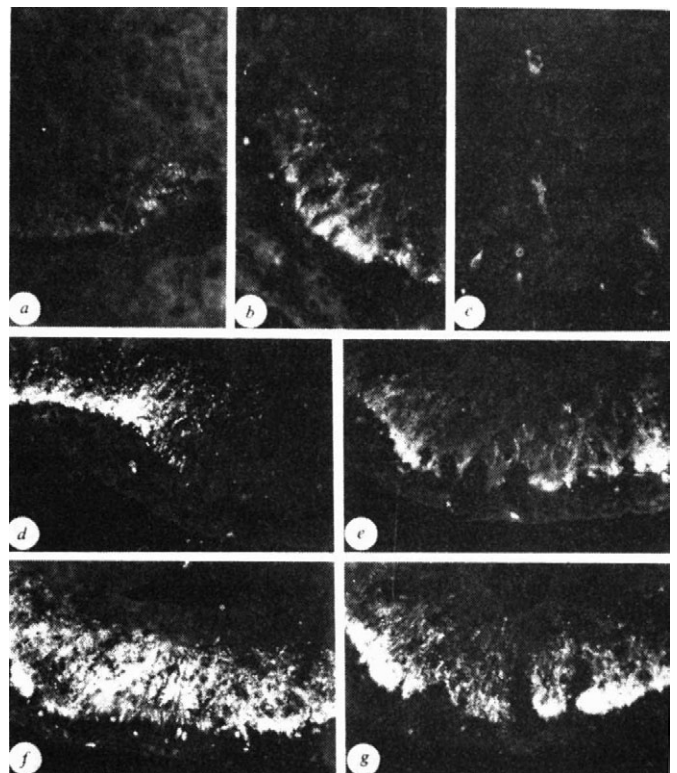


Fig. 2 *a,b*, Anti-CRF-stained fibres and terminals in the median eminences of rat fetuses at day 19 (*a*) and 20 (*b*) of development ($\times 210$). *c-g*, Newborn rat sacrificed more than 12 h after birth. A few perikarya are stained in the paraventricular nucleus (*c*; $\times 210$). Note on adjacent frontal sections of the median eminence, the presence and different distributions of fibres and terminals revealed by antisera to gonadoliberin (*d*), CRF (*e*), vasopressin (*f*), and somatostatin (*g*) ($\times 140$).

angiotensin II-containing neurones^{9,10}. (2) The neuronal system revealed by anti-CRF antiserum is topographically different from those stained with antisera against other neuropeptides (oxytocin, vasopressin, neurophysins, gonadoliberein, somatostatin and pro-opiocortin⁵). (3) During fetal life, the stages of first appearance and the development of these various neuropeptide systems are different (our work in progress).

Results reported here demonstrate a novel hypothalamo-infundibular system, the neurones of which contain a peptide that is immunologically related to CRF. Further evidence indicates that these neurones are involved in the regulation of the corticotrophic axis, which suggests that they contain CRF: total depletion of CLI in these neurones can be obtained after adrenalectomy (our work in progress). Moreover, it has been shown that PVN demonstrate a high degree of CRF activity¹¹ and that lesions of this nucleus reduce the CRF-activity in stalk median eminence extracts¹². Finally, the appearance of CLI-fibres in the median eminence at day 19 of fetal development agrees well with previous encephalotomy and decapitation studies indicating that the hypothalamus controls the pituitary adrenal axis, beginning with the 19th day of fetal life^{13,14}.

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Endogenous opiates regulate oxytocin but not vasopressin secretion from the neurohypophysis

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The neurohypophysis not only contains the nerve endings of the oxytocin- and vasopressin-secreting neurones of the hypothalamus, but also receives both dopamine¹- and opiate peptide^{2,3}-containing nerve fibres. This secondary innervation may regulate hormone secretion at the level of the nerve terminals by an action analogous to presynaptic inhibition⁴. Electrical stimulation of the isolated neurohypophysis releases oxytocin and vasopressin in amounts readily detectable by radioimmunoassay, and presumably also releases dopamine⁵ and endogenous opiates. If these neurosecretory products are indeed regulators of hormone release, then specific antagonists to dopamine and opiates should modify the electrically stimulated release of hormone. We report here that the dopamine antagonist spiperone does not affect the release of either oxytocin or vasopressin from the isolated rat neurohypophysis. In contrast, the opiate antagonist naloxone markedly enhances the electrical stimulation of oxytocin release, but has no effect on vasopressin release. Thus an endogenous opiate, released by electrical stimulation of the isolated neurohypophysis, inhibits the release of oxytocin, but this opiate control does not appear to be exerted on the vasopressin-secreting terminals. We have not found similar evidence that endogenous neurohypophysial dopamine regulates hormone release.

For each experiment the neurohypophysis was dissected from a freshly decapitated male Wistar rat, and impaled on one of a pair of sharpened platinum/iridium stimulating electrodes⁶. The electrode assembly was inserted in a small Perspex chamber, and the gland was continuously perfused at 37°C with oxygenated Krebs medium containing 2 mM CaCl₂, 2.8 mM D-glucose and 0.5 mg ml⁻¹ bovine serum albumin at a rate of 150 µl min⁻¹. Fractions of the perfusate were collected each minute in initial experiments and over longer time periods in subsequent experiments, and the oxytocin⁷ and vasopressin^{6,8} content of the fractions was subsequently determined by radioimmunoassay.

The results of four initial experiments, in which fractions of perfusate were collected each minute, are shown in Figs 1 and 2. After a 30 min wash period, basal secretion of vasopressin and oxytocin was stable in each experiment at 10–60 and 10–30 pg min⁻¹ respectively. Individual glands were then stimulated electrically at 13 Hz (matched biphasic pulses; 2 ms pulse width; 5 mA peak to peak) for 3 min, eliciting peak secretory rates of 160–740 pg min⁻¹ for vasopressin and 170–630 pg min⁻¹ for oxytocin. Ten minutes later the antagonist was introduced into the perfusion medium (10⁻⁶M spiperone;

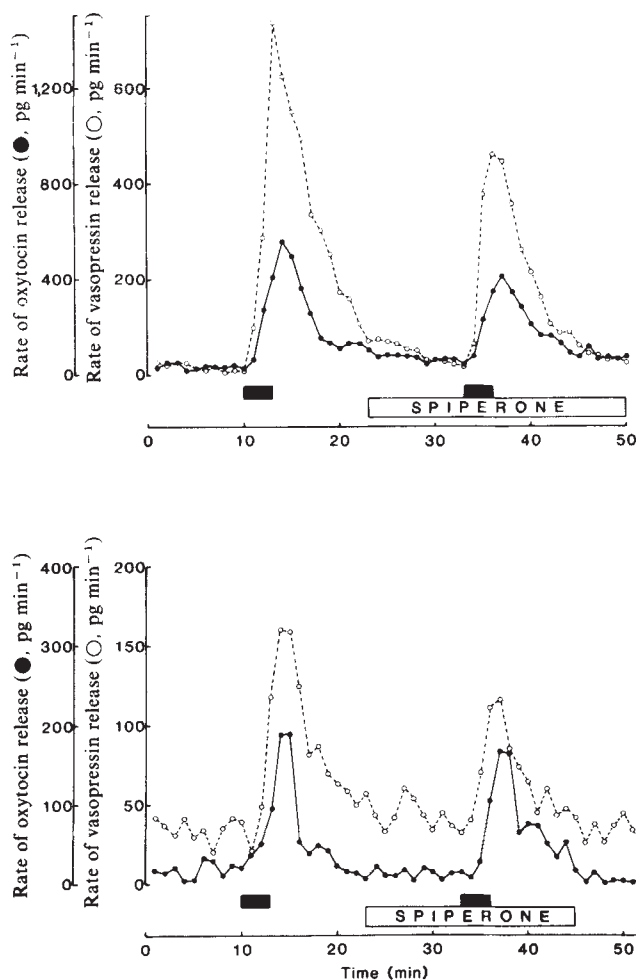


Fig. 1 Hormone release from a single neurohypophysis into continuously perfused medium, in each of two experiments. Vasopressin (open symbols) and oxytocin (solid symbols) were measured by specific radioimmunoassays. Solid bars indicate periods of 13 Hz electrical stimulation; open bar marks the period of inclusion of the dopamine antagonist spiperone (10⁻⁶M) in the perfusate. Spiperone does not markedly influence either basal or stimulated secretion of either hormone. Hormone concentrations are expressed in terms of synthetic arginine-vasopressin (Sigma; designated activity 465 IU mg⁻¹) and synthetic oxytocin (Cambridge Research Biochemicals; measured biological activity 366 IU mg⁻¹).

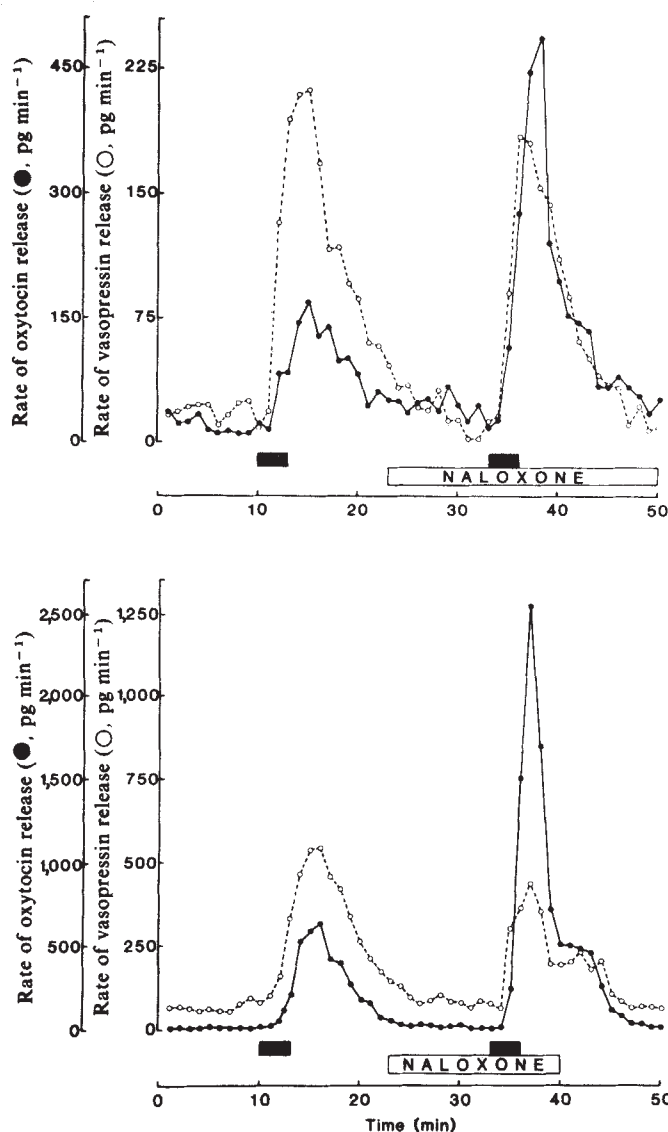


Fig. 2 Effect of the opiate antagonist naloxone (5×10^{-6} M) on vasopressin (open symbols) and oxytocin (solid symbols) release from the isolated neurohypophysis, in each of two experiments. Naloxone causes a marked potentiation of the stimulated secretion of oxytocin, but has no effect on basal or stimulated secretion of vasopressin. Solid bars mark periods of 13 Hz electrical stimulation; open bar marks the period of inclusion of naloxone in the perfused medium.

Table 1 Electrically stimulated release of oxytocin and vasopressin

Test substance	No. of experiments	Vasopressin release S_i/S_c ratio	Oxytocin release S_i/S_c ratio
Control	6	0.883 ± 0.046	0.899 ± 0.090
Spiperone (10^{-6} M)	5	0.773 ± 0.049	0.990 ± 0.053
Naloxone (5×10^{-6} M)	5	0.809 ± 0.069	$1.883 \pm 0.196^*$

S_i/S_c is the ratio of total vasopressin and oxytocin released in the presence of test substance (S_i) to that released in control medium (S_c) by identical electrical stimulations. The protocol was as described for Figs 1 and 2. Data from those experiments are included. Data are mean $\pm$ s.e.m.

* $P < 0.001$ compared with control. No other differences from control are significant (Student's t -test).

5×10^{-6} M naloxone), and after a further 10 min the electrical stimulation was repeated. Most of the hormone released by electrical stimulation in this system appears in the perfusate within 10 min after the end of stimulation (Figs 1, 2). For subsequent experiments, the same stimulus protocol was used, but only total released hormone was measured, by pooling the perfusate collected during the 3 min of stimulation and the 10 min following.

As shown previously⁶, repeated stimulations of the isolated neurohypophysis release progressively less vasopressin as the releasable pool of hormone is depleted, and possibly also as a result of degenerative changes in the isolated gland. In control experiments, the same was true for oxytocin secretion (Table 1). The introduction of spiperone into the perfusion medium had no significant effect on electrically stimulated secretion of either oxytocin or vasopressin (Fig. 1, Table 1). In contrast, naloxone markedly potentiated the electrical stimulation of oxytocin secretion but had no significant effect on vasopressin secretion. In each experiment the oxytocin released by stimulation in the presence of naloxone was 1.5–3 times greater than that released by a prior stimulation, while stimulated vasopressin secretion followed the expected slight decline, as seen in control experiments (Fig. 2, Table 1). We have confirmed that the same holds for the neurohypophysis of female rats, and naloxone also markedly potentiated the stimulated secretion of oxytocin from the neurohypophysis of a Brattleboro rat, homozygous for diabetes insipidus, with an absolute deficiency of vasopressin. Neither antagonist had any significant effect on the basal secretion of either oxytocin or vasopressin (data not shown).

Thus the release of oxytocin by electrical stimulation of the isolated rat neurohypophysis is reduced by the simultaneously evoked release of an endogenous opiate, probably enkephalin or dynorphin. The endogenous opiate does not, however, appear to control the vasopressin-secreting terminals.

Intracerebroventricular injection of opiates inhibits the stimulated secretion of oxytocin *in vivo*, probably by an action on the neurohypophysial nerve terminals. Similarly, enkephalin analogues inhibit the electrically stimulated release of vasopressin from the isolated neurohypophysis^{9,10}, although in these as in our experiments, naloxone had no significant effect on vasopressin secretion. While our results suggest that the opiate innervation of the neurohypophysis is confined in its action to the oxytocin terminals, it remains possible that opiates in the circulation also regulate vasopressin secretion at the level of the nerve terminals. The identity of the endogenous opiate responsible for the regulation of oxytocin secretion is currently under further investigation in our laboratory.

Dopamine appears to be extensively involved in the central control of oxytocin secretion¹¹. As relatively high concentrations of spiperone, a highly specific dopamine receptor antagonist¹², are without effect on basal and stimulated secretion of both oxytocin and vasopressin, the role of the endogenous neurohypophysial dopamine remains to be established.

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Neurotensin in the rat anterior pituitary gland

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Neurotensin, a basic peptide consisting of 13 amino acids, was originally isolated from bovine hypothalamus¹ and subsequently from human and bovine intestine^{2,3}. Radioimmunoassay and immunocytochemical studies have demonstrated that neurotensin-like immunoreactivity (NTLI) is distributed throughout the mammalian central nervous system (CNS)^{4,5} and the intestine, where it is localized to a distinct type of endocrine-like cell^{4,6}. Neurotensin may serve as a neurotransmitter in the CNS⁷ and as a hormone in the gastrointestinal tract^{6,8}. In addition, NTLI has been detected in the rat pituitary gland^{4,5,9}. Here we report that the NTLI present in the anterior pituitary gland of the rat is indistinguishable from synthetic neurotensin, using two chromatographic systems. It is localized in cells from which it can be released by depolarization in a calcium-dependent manner. The neurotensin content of the anterior pituitary *in vivo* was dramatically reduced after 'chemical thyroidectomy' induced by propylthiouracil treatment.

Pituitary samples were dissected and extracted as previously described¹⁰, and NTLI was measured with two radioimmunoassays using rabbit antisera directed against the amino- and carboxy-terminals of synthetic neurotensin¹⁰. The detection limit of each assay was 10 fmol per assay tube. NTLI was detected in anterior pituitary extracts from rat, guinea pig, cow and man, although none was found in pig. Both radioimmunoassays registered similar amounts of NTLI in rat anterior pituitary glands, and the immunoreactivity diluted in parallel with synthetic neurotensin for both antisera.

The NTLI in extracts of rat anterior pituitary was characterized further by gel filtration on a Sephadex G-25 column (Fig. 1a) and reverse-phase HPLC on a C₁₈ μ -Bondapak column (Fig. 1b). With both systems, almost all the immunoreactivity emerged as a single peak in the same position as authentic neurotensin, and could be detected in equivalent amounts using both carboxy- and amino-terminal-directed assays. Both chromatographic systems also separated a second peak of immunoreactivity which accounted for ~10% of the total, and was detectable with both immunoassays. On gel filtration this peak emerged later than synthetic neurotensin, with a V_e (elution volume) of 0.86 (Fig. 1a).

Neurotensin-positive cells were visualized using antiserum directed against the amino-terminal end of the molecule by indirect immunofluorescence^{11,12}. Numerous neurotensin-positive cells were scattered throughout the anterior lobe of the pituitary gland (Fig. 2).

The presence of neurotensin in the anterior pituitary gland prompted us to look for its release from rat hemisected anterior pituitaries. Exposure of the tissue to 40 mM K⁺ led to a marked increase in the basal release of neurotensin (Fig. 3). This release was Ca²⁺-dependent, as high K⁺ produced no increase over the basal rate in the absence of Ca²⁺.

The presence of high concentrations of neurotensin in a specific population of cells in the anterior pituitary suggests a possible role for this peptide in endocrine systems. Therefore, we subjected the hypothalamic-anterior pituitary system to selected stimuli, including surgical adrenalectomy, surgical gonadectomy, dopamine antagonist-induced hyperprolactin-

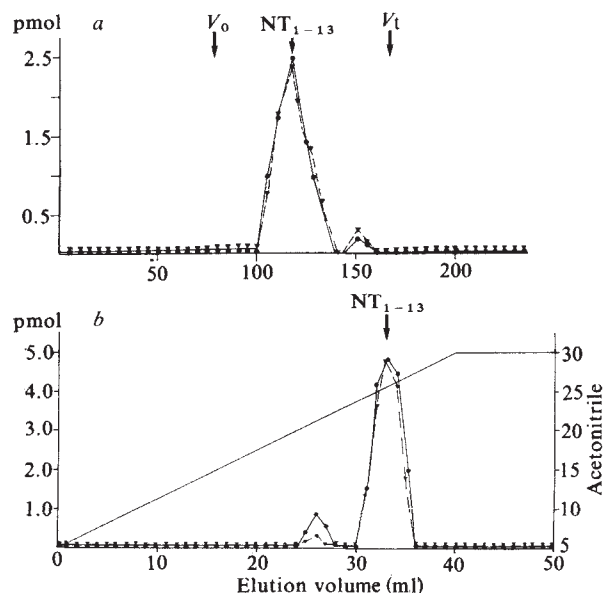


Fig. 1 Fractionation of the neurotensin-like immunoreactivity in acetic acid extracts of rat anterior pituitary gland on a Sephadex G-25 column (a) and a reverse-phase HPLC column (b). a, Lyophilized tissue extracts were reconstituted in 4–5 ml and applied to a Sephadex G-25 column (1.6 × 90 cm), equilibrated and eluted at room temperature with 0.1 M acetic acid. Fractions (3 ml) were collected at a flow rate of 15 ml h⁻¹; these were lyophilized and resuspended in assay buffer using antisera directed against the neurotensin amino-terminus (●) and carboxy-terminus (▼). The column was calibrated by blue dextran (void volume, V_0), synthetic neurotensin (NT₁₋₁₃) and ²²NaCl (total volume, V_t). b, Separation of neurotensin-like immunoreactivity from rat anterior pituitary gland by reverse-phase HPLC carried out as described previously¹⁰. Thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, growth hormone, adrenocorticotrophic hormone, β -endorphin, prolactin, calcitonin, secretin, gastrin, substance P, vasoactive intestinal polypeptide, bradykinin, angiotensin II, TRH and thyroid hormones showed no cross-reaction, even at a 100,000-fold molar excess. Details of the assays, cross-reactivities towards neurotensin, and its amino- and carboxy-terminal fragments have been described previously¹⁰.

aemia, and chemical thyroidectomy. The only procedure that produced marked changes in pituitary neurotensin was chemical thyroidectomy, produced by the administration of propylthiouracil (PTU). Within 1 week of initiation of treatment with PTU, the neurotensin content of the anterior pituitary decreased by >90% (Table 1), and continuation of treatment resulted in a persistent decrease for up to 2 months, the longest time interval studied. The effect of PTU could, however, be prevented by simultaneous treatment with L-triiodothyronine (T₃), or reversed by subsequent treatment with T₃ after the reduction in neurotensin had already occurred (Table 1).

The reversibility of the PTU-induced reduction in anterior pituitary neurotensin concentration by the administration of thyroid hormone strongly suggests that the decrease in neurotensin is due directly to the fall in circulating thyroid hormone caused by PTU. As the hypothalamic response to such a reduction in thyroid hormone concentrations is a compensatory increase in the release of thyrotropin releasing hormone (TRH), we investigated whether the reduction in pituitary neurotensin was a direct result of increased exposure to TRH. Exogenous TRH was given to normal animals through an osmotic mini-pump. Within 4 days of implantation of the minipump, the level of anterior pituitary neurotensin was reduced by ~90%, and remained at this level for the 2 weeks of the TRH infusion (Table 1).

These results confirm the presence of NTLI in the rat anterior pituitary gland, and in the adenohypophysis of various other

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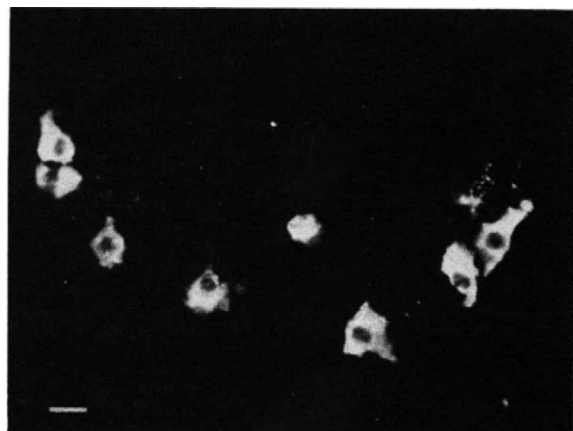


Fig. 2 Cells in the rat anterior pituitary gland containing neurotensin-like immunoreactivity demonstrated by immunofluorescence. Animals were fixed with 4% paraformaldehyde and the pituitaries cut into 25- μ m sections on a microtome¹². Mounted sections were incubated at 4 °C for 24 h with rabbit antiserum raised against the amino-terminus of neurotensin¹⁰ (diluted 1:500). NTLI-positive cells were visualized by subsequent incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (diluted 1:20, 1 h, 25 °C). Fluorescent staining of cells was abolished completely by pre-adsorption of the diluted primary antiserum with 1 μ g ml⁻¹ of synthetic neurotensin. Scale bar, 10 μ m.

mammalian species. Although the exact nature of the NTLI can only be determined by isolating and sequencing the peptide, most of the immunoreactivity in the rat pituitary was chemically indistinguishable from synthetic neurotensin, in two different chromatographic systems. Both separation systems, however, also demonstrated the presence of a second immunoreactive component measurable in both the amino- and carboxy-terminal-directed assays; the chemical nature of this component is unknown.

On a quantitative basis, the anterior pituitary represents one of the richest sources of neurotensin in the rat, the concentration being similar to that in the hypothalamus and ileum⁴. Thus,

Table 1 Effects of manipulations of the hypothalamus–anterior pituitary–thyroid axis on the neurotensin concentration of rat anterior pituitary gland

Treatment	Neurotensin concentration (pmol per g)
a Controls	79 $\pm$ 4.76
PTU	5 $\pm$ 0.22*
PTU + T ₃	71 $\pm$ 4.66
b Controls	76 $\pm$ 2.76
PTU	4 $\pm$ 0.36*
PTU + T ₃	78 $\pm$ 3.96
c Controls	83 $\pm$ 4.45
T ₃	77 $\pm$ 6.88
d Controls	89 $\pm$ 3.66
TRH	4 $\pm$ 0.41*

Adult male Sprague–Dawley rats were given distilled water and put on a low iodine diet containing 0.05% propylthiouracil (PTU) (BP Nutrition). One group of rats (*a*) were given daily subcutaneous (s.c.) injections of either L-triiodothyronine (T₃) (20 μ g per kg) or saline for 12 days. A second group of rats (*b*) were only started on the same dose of T₃ after 12 days of unopposed PTU treatment. The T₃+PTU diet was then administered for the following 21 days. One further group of normal rats (*c*) were given T₃ alone for 1 week. *d*, Thyrotropin-releasing hormone (TRH, Beckman) was administered via a s.c. implanted osmotic mini-pump (Alzet 2001) which delivered TRH at $\sim$ 50 μ g per kg per day; these animals were killed 4 days after mini-pump implantation. Each value represents the mean $\pm$ s.e.m. for eight animals.

* $P < 0.001$.

neurotensin should be added to the growing list of peripheral peptide hormones such as gastrin¹³, secretin¹⁴ and calcitonin¹⁵ that are present in the anterior pituitary. Like β -endorphin^{16,17} and vasoactive intestinal polypeptide^{18,19}, neurotensin has been detected in both the median eminence of the hypothalamus²⁰ and the anterior pituitary gland. The high concentration of NTLI in the median eminence suggests that neurotensin may also act as a releasing factor for anterior pituitary hormones, whereas its localization in anterior pituitary cells and calcium-dependent release by K⁺ suggest a peripheral hormonal role. Several studies have indicated an effect of neurotensin on anterior pituitary hormone secretion²¹, although so far no peripheral endocrine effects have been demonstrated.

The nature of the neurotensin-positive cells in the anterior pituitary is of great interest. We do not know whether these cells also contain any of the known anterior pituitary hormones. The pronounced effects of either hypothyroidism or TRH

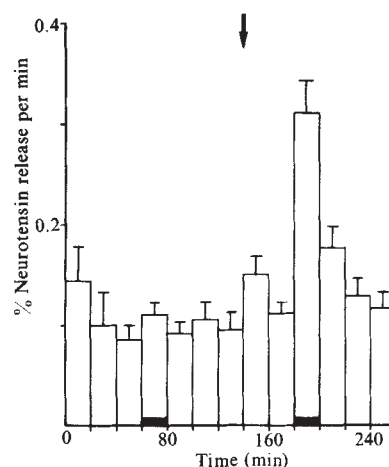


Fig. 3 Ca²⁺-dependent release of neurotensin-like immunoreactivity from rat anterior pituitary evoked by depolarizing concentrations of K⁺. Male Sprague–Dawley rats (150–200 g) were killed and the hemisected anterior pituitaries transferred to a pre-warmed, oxygenated Krebs–bicarbonate solution (composition in mM: NaCl, 125; KCl, 2.5; KH₂PO₄, 1.2; MgSO₄ 1.2; NaHCO₃, 25; D-glucose, 10; EDTA, 0.03; ascorbic acid, 0.6; bacitracin, 0.03, maintained at 37 °C and saturated with 95% O₂/5% CO₂ throughout). Six anterior pituitaries ($\sim$ 60 mg of tissue) were transferred to plastic chambers containing 2 ml of Krebs solution and incubated at 37 °C under constant oxygenation for 20-min intervals. At the end of each interval, the incubation medium was removed onto ice, and NTLI was determined in an amino-terminus-directed radioimmunoassay using a neurotensin standard curve prepared in the same Krebs solution. At the end of the experiment, the tissue was collected, extracted in 0.1 M HCl, lyophilized and NTLI determined in a separate assay. The results are expressed as fractional release rate constants (defined as the per cent total tissue NTLI released per min) and represent the mean $\pm$ s.e.m. ($n = 6$). Solutions to the left of the arrow contained 0.5 mM EGTA and no Ca²⁺, those to the right contained no EGTA and 1.8 mM CaCl₂. The black bars indicate the replacement of 40 mM NaCl with 40 mM KCl. Standard curves for neurotensin in all these media were superimposable.

administration on the neurotensin levels of the anterior pituitary suggest the existence of a functional relationship between the two systems, although the nature of this relationship is not clear. Hypothyroidism leads to an increase in release of TRH from the hypothalamus, which in turn may either release neurotensin from the anterior pituitary, without affecting its synthesis, or inhibit neurotensin synthesis. Interestingly, a functional antagonism by TRH of certain biological effects of neurotensin, such as hypothermia²², muscle relaxation²³ and antinociception²⁴ has been observed.

Thus, the present results indicate that, apart from its possible roles as a neurotransmitter in the CNS and as a gastrointestinal

hormone, neurotensin is also an anterior pituitary peptide which may exert effects both within the pituitary gland itself and on peripheral tissues.

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Do protons block Na⁺ channels by binding to a site outside the pore?

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In a detailed structural model of the Na⁺ channel which has evolved over the past decade, the selectivity of the channel for cations^{1,2}, the voltage-dependence of channel block by protons³ and other small cations², and block by tetrodotoxin (TTX)^{4,5} all depend on the presence of a negatively charged site inside the channel that is part of a constriction called the 'selectivity filter'. Much of the detail inherent in the model arises from Woodhull's quantitative description of the voltage dependence of Na⁺ channel block by protons³, in which the apparent relief of block observed at positive potentials occurs because the blocking site is located inside the sodium channel ~25% of the way through the membrane voltage drop. I report here that proton block of skeletal muscle Na⁺ channels, determined from tail currents, is voltage-independent, and that the apparent voltage-dependent block of peak Na⁺ currents is instead an effect of low pH on channel kinetics. These results suggest that the proton binding site lies effectively outside the membrane field and thus at or outside the channel mouth. As a consequence, present models of the channel interior will need to be re-examined and substantially modified.

Figure 1 illustrates the basic observation, confirmed by others in a variety of tissues^{6–11}, from which it was concluded that

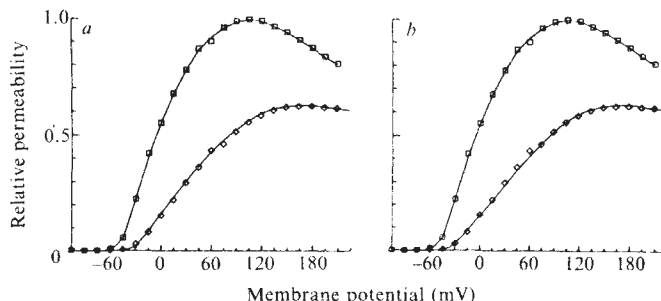


Fig. 1 Proton block of peak Na⁺ channel permeability. Relative permeabilities at the peak of Na⁺ current are plotted against voltage. The Vaseline gap technique was used to voltage-clamp single fibres from the bullfrog *Rana catesbeiana*. External solutions contained (mM) 110 Na, 5 Cs, 2 Ca and 10 HEPES (pH 7.3) or maintenance electrolyte solution (MES) (pH 5.9 and 5.0). Fibre ends were cut in CsF (110 mM), NaF (10 mM) and HEPES (10 mM), pH 7.3. The CsF effectively blocks delayed rectifier currents at all but the strongest depolarizations. At very strong depolarizations, some delayed outward current is seen, but it is generally too small and activates too slowly to interfere with measurements from Na⁺ channels. A combination of standard analogue and digital subtraction procedures eliminated linear leakage and capacity currents. Series resistance compensation was typically set at 3 kΩ. The holding potential was set at -120 mV, temperature 4.5 °C, muscle 173. Data points and the smooth curve through the pH 7.3 points are identical in *a* and *b*. □, pH 7.3; ◇, pH 5.0. The simplified version of Woodhull's theory fitted is, in which protons do not pass through the channel. The fraction (*p*) of channels free from block is given by:

$$p = 1/(1 + [H^+]/K_d) \quad (1)$$

and

$$K_d = K_d(0 \text{ mV}) \exp(\delta EF/RT) \quad (2)$$

where *K_d* is a voltage-dependent dissociation constant, *E* is the membrane potential, *K_d*(0 mV) is the dissociation constant at 0 mV, *F*/*RT* = 1/24 mV at 5 °C, and *δ* is the fraction of the membrane voltage drop traversed by protons to reach the blocking site. In addition, low pH solutions cause an apparent shift in the voltage dependence of channel kinetics. Thus, two additional parameters are typically required to fit peak permeabilities measured at low pH: the amount of voltage shift and a scale factor to account for the presumed change in long-term inactivation caused by this shift. The control curve through pH 7.3 points was drawn by eye and entered into the computer. Curves through pH 5.0 points were derived from the control curve using equations (1) and (2). *a*, pH 5.0 points fitted with *δ* = 0.25, *pK_d*(0) = 5.4, shift = 10 mV, and with the control curve scaled as if there were 10% more channels available for opening at the resting potential at pH 5.0 than at pH 7.3. In essence, long-term inactivation as used here and in previous studies^{3,8} represents a fourth free parameter to be adjusted for a fitting. The figure demonstrates that using this fourth parameter it is possible to fit the peak permeabilities at pH 5.0 with the same values of *δ* and *pK_d*(0) determined in those studies. *b*, Contrary to the assumptions used in the fit in *a*, control experiments have shown long-term inactivation to be 97–100% removed at the holding potential of -120 mV used in these experiments. Thus in *b*, long-term inactivation has been eliminated as a free parameter, and pH 5.0 points are fitted well with *δ* = 0.17, *pK_d*(0) = 5.1, shift = 10 mV. Muscle 173.

proton block of Na⁺ channels is voltage-dependent. At low pH, the reduction of peak Na⁺ channel permeability is greater for small depolarizations than it is for large depolarizations. Figure 1 shows two fits of Woodhull's model, and demonstrates that there is considerable interaction among the fitted parameters. Thus, in Fig. 1*a*, parameters almost identical to those previously reported in nerve and muscle can be obtained if it is assumed, as in previous reports^{3,8}, that low pH causes a decrease in long-term inactivation¹². However, this assumption is not appropriate in the present experiments because the holding potential of -120 mV eliminates almost all long-term inactivation. When this additional constraint is applied, the equally good fit shown in Fig. 1*b* is obtained. The resulting value of *δ* = 0.17, is rather less than *δ* = 0.25 previously reported in frog nerve and muscle.

Determination of block from measurements of peak permeability as in Fig. 1, does not test the theory at negative potentials where the Woodhull model and subsequent modifications of it^{10,13} predict that proton block will be enhanced. For example, at pH 5.9, the model (equations (1) and (2) with *δ* = 0.17 and *pK_d*(0) = 5.1) predicts that 25% more channels will be blocked at -135 mV than are blocked at +15 mV. At pH 5.0, the prediction is that 2.1 times as many channels will be blocked

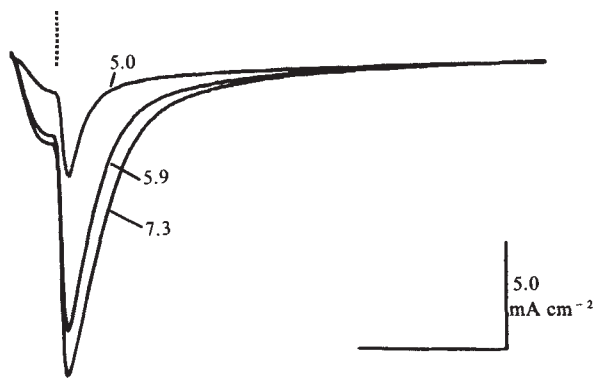


Fig. 2 Na^+ channel tail currents at normal and low pH. Na^+ currents were elicited with a two-pulse voltage-clamp protocol. During the prepulse to +15 mV (left of broken line) and the test pulse to -135 mV, currents in low pH solution were reduced by similar ratios. Ordinarily, Na^+ channels are not open at potentials negative to -50 mV. The purpose of the two-pulse protocol was open Na^+ channels with a depolarizing prepulse and then to step to various test voltages where currents were measured before the channels closed. The horizontal calibration bar represents 0.5 ms during the test pulse and 2.5 ms during the prepulse. The holding potential was -105 mV, temperature 4.6°C. Muscle 149.

at -135 mV. The records shown in Fig. 2 suggest that this is not the case for frog skeletal muscle. Instead, currents elicited by the prepulse to +15 mV are reduced at low pH by about the same ratio as the tail currents elicited by the test pulse to -135 mV, suggesting that block is voltage-independent. This point is demonstrated quantitatively, over a wide range of test voltages, by the isochronal permeability-voltage relationships shown in Fig. 3a, b. The continuous curves fitted to the low pH data were computed from the control curve assuming that: (1) block is independent of voltage; (2) low pH slightly shifts the voltage dependence of tail current kinetics; and (3) the apparent pK_d of the blocking site depends on the solution pH. These points will be considered in turn.

In general, data were well fitted assuming block to be independent of voltage (equation (1) with K_d constant). Different isochronal intervals produce permeability-voltage relationships having different shapes but do not alter the fitted parameters of proton block. Replacing half of the sodium outside the fibre with the impermeant tetramethylammonium ion also changes the shape of the isochronal permeability-voltage relationship without significantly altering fitted parameters of block. In some fibres a small degree ($\delta < 0.03$) of voltage-dependence improved the fits slightly at extreme positive and negative potentials. In two fibres this apparent voltage dependence of K_d was accompanied by larger than normal delayed rectifier currents. In other fibres best fits obtained early in the experiment required no voltage dependence, whereas later runs on the same fibre exhibited increasing apparent voltage dependence as leakage current increased. Therefore, although a small degree of voltage dependence may exist, it seems more likely to be due to an artefact unrelated to block of Na^+ channels. In contrast to fits in which K_d is independent of voltage, the dotted curve in Fig. 3b illustrates a fit using the Woodhull theory and parameters determined in Fig. 1. The fit is particularly poor at negative voltages where the voltage-dependent theory predicts enhanced block. The complete Woodhull theory, which allows protons to pass through the channel, produces a fit which is better at one or two of the most negative potentials but which is worse overall than the dotted curve shown.

The isochronal permeability measured at any voltage is dependent on the rate of decay of the tail current. Included in the fits is a shift of the permeability-voltage relationship to account for a voltage shift in the kinetics of tail currents. The average shift, in the depolarizing direction, was 3.4 ± 2.3 mV ($n = 10$) at pH 5.9 and 16.6 ± 7.4 mV ($n = 11$) at pH 5.0. These

shifts are similar to kinetic shifts previously reported in nerve and muscle^{3,8-11,14-16} and to shifts determined from exponential fits to tail currents at normal and low pH in the present experiments (not shown). Applying this voltage shift assumes that permeability, as calculated from the constant field equation^{17,18}, provides a good description of the current-voltage relationship of open Na^+ channels in this preparation. The instantaneous current-voltage relationship in frog skeletal muscle shows more curvature than exhibited by the constant field equation⁸. However, the instantaneous permeability-voltage relationship is essentially flat between 0 and -105 mV and only slightly voltage-dependent at positive potentials. Thus, for the small voltage shifts applied here, the constant-field description does not introduce significant errors in the fitted parameters.

Fitted values of pK_d were found to depend on the pH of the test solution. Thus, at pH 5.9 the permeability was reduced by 23% for an apparent pK_d of 5.37 ± 0.19 (mean $\pm$ s.d., $n = 10$), whereas at pH 5.0 the permeability was reduced by 33% for an apparent pK_d of 4.69 ± 0.16 ($n = 11$). As previously mentioned, the holding potential used in these experiments makes it unlikely that this dependence of K_d on pH is due to an effect of low pH on long-term inactivation. Another explanation is interaction between the blocking site and another nearby protonatable site. Reducing the pH from 5.9 to 5.0 might promote greater occupancy of the neighbouring site and by coulombic interaction thereby alter the affinity of the blocking site.

The seemingly contradictory results of Figs 1 and 3 can be reconciled if the effect of low pH on channel kinetics is more complex than a simple shift in voltage dependence. In amphibian myelinated nerve, it has been reported that at normal pH the number of channels open at the peak of current is constant for depolarizations $\geq +30$ mV. Figure 4 demonstrates that this is not true in frog muscle. Instead, the number of channels open at the peak of current continues to increase with

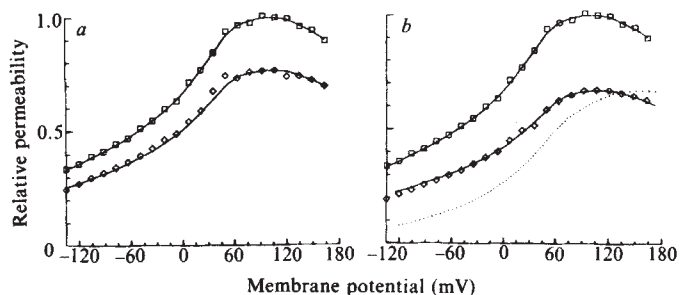


Fig. 3 Isochronal permeabilities measured from Na^+ tail currents. Tail currents were measured with a two-pulse protocol similar to that used in Fig. 2 except that prepulses were to +90 mV, and the holding potential was -120 mV. The tail currents shown in Fig. 2 have nearly exponential time courses; however, at more positive potentials the tail time course is a complex mix of activation and inactivation kinetics. This complication prevents simple extrapolation of measured currents back to the time of the test step used to measure the so-called 'instantaneous' permeability-voltage relationship. Instead, the illustrated isochronal permeability-voltage relationships were calculated from the current measured 60 μ s after the step to the test pulse, using the constant field equation. The shape of this isochronal permeability relationship is determined by both the effect of voltage on tail current kinetics and by the current-voltage relationship of open Na^+ channels. *a*: $\square$, Control curve (pH 7.3) drawn by eye and entered into the computer; $\diamond$, curve through pH 5.9 points, fitted to the data by scaling the control curve by equation (1) with $pK_d = 5.4$, independent of voltage. The scaled curve was shifted 3 mV in the depolarizing direction. *b*, Isochronal permeability-voltage relationships at pH 7.3 and 5.0. The points for pH ($\square$) and for the control curve were identical to those in *a*. The smooth curve through the pH 5.0 points ($\diamond$) was fitted with a voltage-independent pK_d of 4.68 and a shift of 10 mV. The dotted curve is the control curve scaled by the Woodhull theory with $\delta = 0.17$ and $pK_d(0) = 5.1$ as in the fit of Fig. 1b. Temperature, 3.6-4.2°C. Muscle 158.

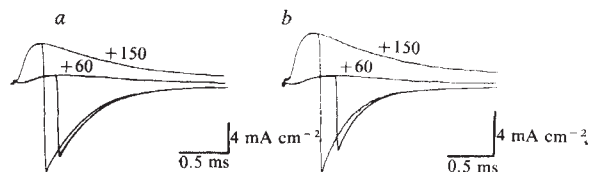


Fig. 4 Relative number of channels open at the peak current, at normal and low pH. *a*, At pH 7.3, pulses to +60 and +150 mV were interrupted at the peak of current and the voltage stepped in both cases to -75 mV. The prepulse of +150 mV elicited a tail current 11% greater than the prepulse of +60 mV, implying that 11% more channels are open at the peak at +150 mV. *b*, Currents from a fibre bathed in pH 5.0 Ringer's using the same pulse protocol as in *a*. The prepulse to +150 mV elicited a tail current 39% greater than the prepulse to +60 mV. Between +60 and +150 mV, the relative increase in the number of channels open at the peak of current was thus 26% greater at pH 5.0 than at pH 7.3. This is the same relative increase as that seen between +60 and +150 mV in Fig. 1*b*. Muscle 173, holding potential -120 mV, temperature 4.7 °C.

increasing depolarization at both pH 7.3 and 5.0, but at low pH this increase is considerably steeper. Between +60 and +150 mV, this effect of pH on the voltage-dependence of channel opening quantitatively accounts for the value of $\delta = 0.17$ found in the fit shown in Fig. 1*b*. These results conflict with the results of Woodhull in frog nerve which suggest that over this voltage range the number of channels open at the peak of current is constant. However, the differences between my results and those of Woodhull may represent kinetic differences between frog nerve and muscle. The experiment illustrated in Fig. 4 needs to be repeated in myelinated nerve using computer subtraction of capacity transients and with the faster voltage-clamp circuits now available for the node of Ranvier.

Thus, I have reported two effects of low pH on the Na⁺ channels of frog muscle which are consistent with previous observations in amphibian nerve and muscle, but which lead to different conclusions about the structure and function of the channel. The first concerns the structure of the open Na⁺ channel and the localization of the site of proton block. In the Hille model of the Na⁺ channel 'selectivity filter', a negatively charged site stabilizes the partially dehydrated ion in its traverse of the narrowest constriction of the channel. Tetrodotoxin and protons are hypothesized to block at this same site, which because of Woodhull's results, was proposed to be located inside the channel about 25% of the way down the membrane voltage drop. I have presented evidence that proton binding to the blocking site is independent of voltage; for this to be true, the site must lie outside the membrane field. Unless some part of the Na⁺ channel interior is also effectively outside the field, the blocking site must be at or near the outside of the channel. These results suggest that a new model of Na⁺ channel structure is required. Spalding has demonstrated¹⁹ that treatment of muscle fibres with the carboxyl-modifying reagent trimethyl-oxonium ion (TMO) reduces the sensitivity of Na⁺ channels to TTX without altering channel selectivity and without eliminating proton block. His results also support the notion that there is a second ionized acid site near the proton blocking site and that modification of this second site may alter the affinity of the blocking site. Thus, it may be necessary to hypothesize separate sites for ionic selectivity, TTX binding and proton block.

Second, concerning the effect of low pH on channel kinetics, the number of channels open at the peak of current is not constant for strong depolarizations, but continues to increase over the entire voltage range examined. At low pH this increase is more strongly voltage-dependent than at normal pH. Thus, the kinetic effect of low pH cannot be explained by previous theories which suggest that low pH changes surface potential, thereby producing a simple shift in the voltage-dependence of channel gating.

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Note added in proof: Mozhayeva *et al.*²⁰ report that in frog nerve proton block of tail currents is also less at negative potentials than predicted by the simplified Woodhull theory. Because their measurements do not extend to potentials more negative than -50 mV, a more specific comparison with my results is not possible.

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Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins

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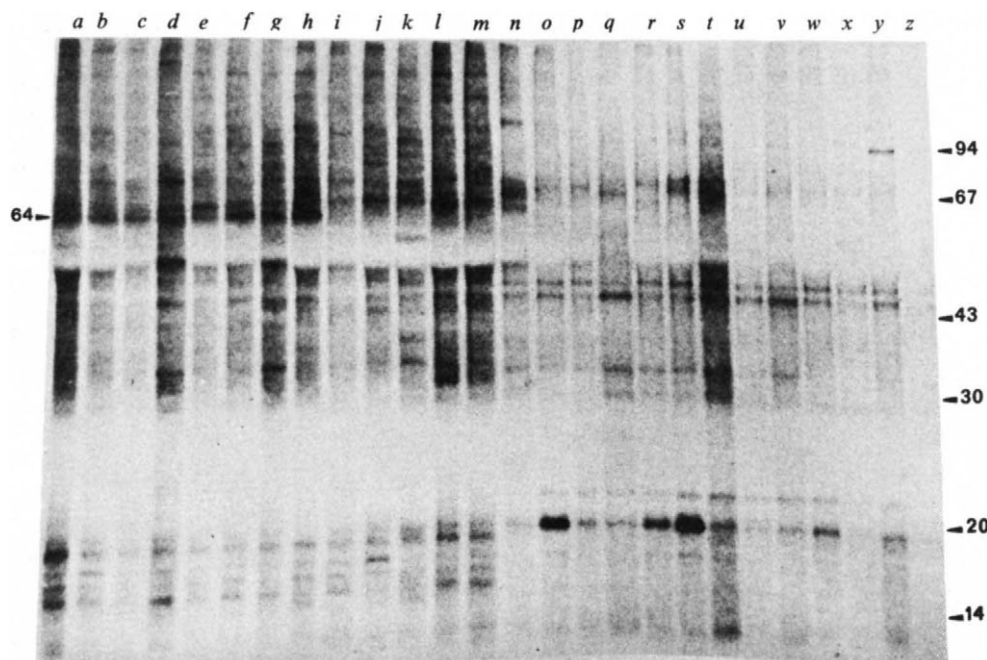
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Insulin-dependent diabetic (IDD) patients have a high prevalence of circulating autoantibodies against islet of Langerhans cells at the time of diagnosis¹⁻⁴. Inflammatory cells within the islets⁵, leukocyte migration inhibition in response to pancreatic antigens⁶ and an association with certain HLA-D/DR histocompatibility antigens^{7,8}, have also been observed. It seems that the autoantibodies may be pathogenically relevant as they react primarily with β -cells⁹, but the specific target antigen(s) have yet to be identified. In the present study we determined whether sera from insulin-dependent diabetic children are able to immunoprecipitate proteins from detergent lysates of human islet cells. We report that sera from 8 out of 10 newly diagnosed diabetic children consistently immunoprecipitate a protein having a molecular weight (M_r) of ~64,000 (64K). An additional protein (38K) was precipitated from islet cells obtained from a HLA-DR3-positive donor. Neither of the proteins was precipitated by non-diabetic sera nor detected in immunoprecipitates from human lymphocyte lysates. It is suggested that the 64K and/or 38K protein components may represent cell-specific target antigens in insulin-dependent diabetes.

Human islets of Langerhans were isolated from the pancreas of five cadaver kidney donors. The age, sex and HLA-typing of the donors are shown in Table 1. Portions of pancreas (~2-3 g) were treated with collagenase^{10,11} to digest all exocrine tissue. Following washing by centrifugation, islets were individually selected using a stereomicroscope. The islets were maintained in culture for 1-5 days and the production of insulin was demonstrated by radioimmunoassay¹².

Islets were biosynthetically labelled with ³⁵S-methionine and lysed in 1% Nonidet P-40 (NP40) (see Fig. 1 legend). The

Fig. 1 Immunoprecipitation of human islet cell (pancreas donor 1; lanes a-m) and lymphocyte (lymphocyte donor 1; lanes n-z) proteins with human sera. Lanes a-j and n-w represent serum samples from newly diagnosed diabetic children (1-10); lanes k, l and x, y, sera from two individual controls; and lanes m and z, a pool of serum samples from five normal children. Islets (200 per ml) were labelled for 16 h, and peripheral lymphocytes from a healthy male donor ($1 \times 10^7 \text{ ml}^{-1}$) for 6 h, in methionine-free Eagle's minimal essential medium (pH 7.4) with Earle's salts, 1.0 g sodium bicarbonate, 5% fetal calf serum, 100 units ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, 16.7 mM glucose and ^{35}S -methionine (200 $\mu\text{Ci ml}^{-1}$). Islets and lymphocytes were washed twice at 4°C in medium containing 5 mM methionine and once in buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 5 mM methionine, 1,000 KIE ml^{-1} trasylol and 2 mM phenylmethylsulphonyl fluoride (PMSF); and were then lysed in the same buffer containing 1% NP40 (v/v) (NP40 buffer). Insoluble material was removed by centrifugation (30 min at 100,000g). The lysate was incubated with normal human serum (10 μl per 100 μl supernatant) followed by adsorption to an excess of formalin-fixed heat-inactivated *Staphylococcus aureus* (Cowan I strain) before immunoprecipitation with diabetic serum. Aliquots (100 μl , 2×10^7 c.p.m.) of preabsorbed lysate were incubated with 25 μl diabetic sera or control sera for 3 h. To each assay tube, 100 μl of pre-swelled protein A-Sepharose CL-4B (Pharmacia) were added and the reaction mixture was incubated at 4°C for a further 30 min. The protein A-Sepharose was then washed four times by centrifugation in 0.5% NP40 buffer and once in cold water. The immune complexes were eluted by boiling for 3 min in sample buffer containing 80 mM Tris (pH 6.8), 3% (w/v) SDS, 15% sucrose, 0.001% bromophenol blue and 5% (v/v) β -mercaptoethanol. The Sepharose beads were removed by centrifugation and the supernatant analysed by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide slab gel. The gel was stained and processed for autoradiography as described elsewhere²¹.



cytoplasmic extracts were precipitated with normal human serum before immunoprecipitation with diabetic sera. Resulting immune complexes were isolated by a protein A immunosorbent¹³ and the labelled antigens identified by autoradiography after SDS-polyacrylamide gel electrophoresis (SDS-PAGE)¹⁴ (Figs 1, 2). Lymphocytes, separated from the blood of four healthy individuals by density gradient centrifugation¹⁵ were labelled and processed in parallel with the human islets (Table 1).

Sera from 10 IDD patients (4 females and 6 males, 11-16 years old) and was obtained 2-9 days after diagnosis. HLA-typing showed that 8 patients were HLA-DR3,4; one was HLA-DR3 and another was HLA-DR1,4-positive (Table 2). All patients were treated with highly purified porcine insulin (NOVO A/S, Denmark) and had no insulin antibodies. Sera from each of six healthy individuals were used as controls.

Sera from 8 out of 10 IDD patients immunoprecipitated a band of ~64K (Fig. 1, lanes a-h) from pancreas donor 1 and 9 out of 10 sera precipitated the 64K component of pancreas donor 2 (Table 2). Islet lysate prepared from donor pancreas 3 was tested with six sera, all of which immunoprecipitated the 64K component (Table 2) and islet preparations from donors 4 and 5 were each tested with four sera, all of which reacted with the 64K component (Table 2; Fig. 2a-d).

Control sera were consistently negative (Fig. 1k-m; Fig. 2e-f) and neither the 64K nor a specific lymphocyte protein was detected by the diabetic sera (Fig. 1n-z). All four diabetic sera tested on pancreas 5, which was HLA-DR3-positive, precipitated a 38K component in addition to the 64K component (Fig. 2a-d; Table 2). This protein was not precipitated by control sera (Fig. 2e-f) nor was it detected in lymphocytes (data not shown).

As several studies have shown that islet cell antibodies are species nonspecific^{3,4} we prepared lysates from mouse islets (NMRI, A.TH and CBA/H mice), from rat insulinoma cells¹⁶ and from mouse and rat spleen lymphocytes. Serum samples from five of the diabetic children, all precipitating the 64K protein from human islets, were tested. In contrast to the results with human islet cell material the results were highly variable. A protein of M_r 64K was precipitated by 1-2 diabetic sera

tested on islet lysates of the three different strains of mice. A protein of 59K was precipitated by two diabetic sera from lysates of A.TH or CBA/H mouse islets, while a 40K component was precipitated by all four diabetic sera tested on NMRI mouse islets. One serum tested on rat insulinoma cell lysates, detected a 64K component in 5 out of 10 individual tumours.

Our study demonstrates that sera from children with newly diagnosed insulin-dependent diabetes can contain antibodies which recognize at least one human islet cell protein. This antigen was detected in islet lysates of all five pancreas donors. It was also observed that all four serum samples tested immunoprecipitated another component from a HLA-DR3-positive pancreas donor. These antigens were not recognized by control serum nor were they detected in lymphocytes.

Sections of human blood group O pancreas^{1,3} were used to determine islet cell (cytoplasmic) antibodies (ICA) in indirect immunofluorescence tests detecting either human IgG (IgG-ICA) or human complement component C3 (complement-fixing or CF-ICA)¹⁷. In addition, dispersed NMRI mouse⁴ or rat islet cells⁹ were used to detect islet cell surface antibodies by immunofluorescence (Table 2). The analysis in Table 2 suggests that all 10 diabetic sera contained antibodies detectable in one

Table 1 Age, sex and HLA-type of pancreas and lymphocyte donors

Pancreas donor	Age	M/F	A	HLA-type			DR
				B	C		
1	45	F	3, 33	14, 49	w8		1, 4
2	18	M	1, 2	17, 44	w4		1
3	18	M	2	44	w2		1, 2
4	33	M	3, w32	13, 40	w3, 6		4
5	58	F	1, 25	8, 35	w4		3
Lymphocyte donor							
1	26	M	1, w30	8, 13	w6		3, 7
2	31	M	2, 3	14, 40	w3, 8		w6
3	36	M	2, 3	7, 44	—		4
4	32	M	w26, w33	5, 14	w8		5

Table 2 Immunoprecipitation of human islet cell proteins by diabetic sera

Patient	M/F	HLA-DR	1	2	3	4	5	IgG-ICA	CF-ICA	Mouse ICSEA	Rat ICSEA β -cells	Non- β -cells
1	F	3, 4	64K	64K	NT	64K	64K, 38K	1/3	0/2	+	+	+
2	M	3, 4	64K	64K	64K	NT	64K, 38K	2/3	1/2	+	+	—
3	M	3, 4	64K	64K	64K	64K	64K, 38K	0/3	0/2	—	+	—
4	M	3, 4	64K	64K	64K	64K	64K, 38K	3/3	1/2	+	+	—
5	F	3, 4	64K	64K	64K	64K	NT	3/3	2/2	—	+	—
6	F	3, 4	64K	64K	64K	NT	NT	3/3	2/2	+	+	—
7	M	3, 4	64K	64K	64K	NT	NT	3/3	2/2	—	—	—
8	F	3, 4	64K	64K	NT	NT	NT	3/3	0/2	—	+	—
9	M	3	—	—	NT	NT	NT	3/3	1/2	+	—	—
10	M	1, 4	—	64K	NT	NT	NT	3/3	0/2	+	+	—
n = 10	6/4		8/10*	9/10	6/6	4/4	4/4	—				

M/F is male-female ratio. NT, not tested. Islet cell cytoplasmic antibodies (ICA) determined by indirect immunofluorescence with either antibodies against human IgG (IgG-ICA) or human C3 (complement-fixing or CF-ICA) are shown as number of positive sera versus number of independent laboratories analysing the samples. Islet cell surface antibodies (ICSEA) were tested by indirect immunofluorescence* on dispersed NMRI mouse islet cells or on rat cells enriched in β -cells and non- β -cells⁹.

* Number of 64K and 38K (pancreas 5) protein-precipitating sera versus number of sera tested.

or more of these assay systems. The use of these different methods of antibody detection may further increase the number of patients having immunological markers of insulin-dependent diabetes. Further studies should clarify possible correlations between the different methods¹⁸.

It was noted that the components precipitated from human islets seem to represent minor islet cell proteins, as they constituted approximately 0.02% of the total radioactivity precipitable with 10% trichloroacetic acid.

The results with immunoprecipitation of lysates of mouse pancreatic islets and rat insulinoma cells and the immunofluorescence of mouse and rat islet cells, support previous suggestions that the islet cell autoantibodies are cell- but not species-specific^{3,4}. However, the cross-reactivity with labelled rodent antigens was highly variable, suggesting that human pancreatic islets are required for reliable determination of islet cell antibodies in man. Differences in autoantibody specificity and/or binding capacity may explain the variable reactivity of the antibodies not only between but also within species. The observation of a 38K component precipitated from

HLA-DR3-positive donor islets is intriguing, but further studies are needed to determine whether islet cells from DR3-positive individuals contain a unique antigen recognized by diabetic sera.

The present system does not distinguish between intracellular and cell surface antigenic components nor does it allow us to conclude that the antigens are present solely in β -cells. To test whether islet cell surface antibodies in the diabetic children affect β -cell function, we have perfused rat islet cells¹⁹ with an immunoglobulin fraction from patients 1, 3, 4, 5, 6 and 7. Compared with immunoglobulin from controls, all six preparations inhibited glucose-induced insulin release²⁰, suggesting the presence of antibodies which interfere directly with rat β -cell function.

Several disorders in man are associated with circulating autoantibodies. The autoantibodies often react with cells or tissues from healthy donors but the antigenic determinants have not been characterized. The isolation and characterization of specific islet cell autoantigens would be important from the point of view of developing specific and sensitive immunoassays to monitor autoantibodies among diabetic patients as well as susceptible individuals. Our demonstration that islet cell proteins can be immunoprecipitated by diabetic sera suggests there is an islet cell autoantigen in insulin-dependent diabetes. The methodology used should be applicable to other autoimmune disorders in which the antigens have not been identified.

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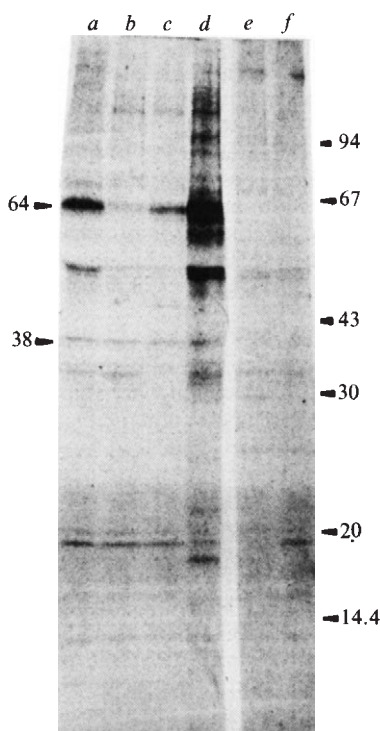


Fig. 2 Immunoprecipitation of human islet cell proteins from pancreas donor no. 5 with diabetic sera 1-4 (lanes a-d) and sera from two individual controls (lanes e, f). Experimental details were as described in Fig. 1 legend except that 100 μ l aliquots of preabsorbed islet cell lysate were incubated with 50 μ l diabetic sera or control sera followed by adsorption to *S. aureus* (Cowan I strain).

Hybrids between rat lymphoma and mouse T cells with inducible cytolytic activity

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Cytolytic T lymphocyte (CTL) proliferation is dependent on T-cell growth factor (TCGF). However, fusion of mouse CTLs with T lymphoma cells yields two types of hybrids, cytolytic ones in which proliferation remains dependent on TCGF and others which express neither this dependence nor cytolytic function¹. Clones of the first type can generate variants of the second type. TCGF-independent hybrids² remain non-cytolytic when cultured in medium supplemented with supernatant of concanavalin A (Con A)-stimulated rat spleen cells (CS) which is our standard source of TCGF. Here we report that in TCGF-independent hybrids between a murine CTL line and a rat T lymphoma, high cytolytic activity can be induced by the addition of CS to the medium. The induction is reversible and seems to depend on a factor in CS which is different from TCGF and from immune interferon.

B6.1.SF.1 is a cloned murine CTL line derived from a female C57BL/6 mouse maintained in culture for more than 2 yr³. At the time of fusion the line had high cytolytic activity against the BALB/c myeloma S194 but it had lost its original activity against H-2^d lipopolysaccharide blast target cells. It has a near-diploid karyotype with one abnormal metacentric chromosome⁴. W/Fu(C58NT)D is a rat T lymphoma line⁵, a thioguanine- and ouabain-resistant mutant of which was obtained from R. Hyman (Salk Institute). It has a near-diploid karyotype containing 22 submetacentric or metacentric and 18 telocentric chromosomes.

B6.1.SF.1 and W/Fu(C58NT)D were fused and plated in medium with or without CS (for details see Fig. 2 legend).

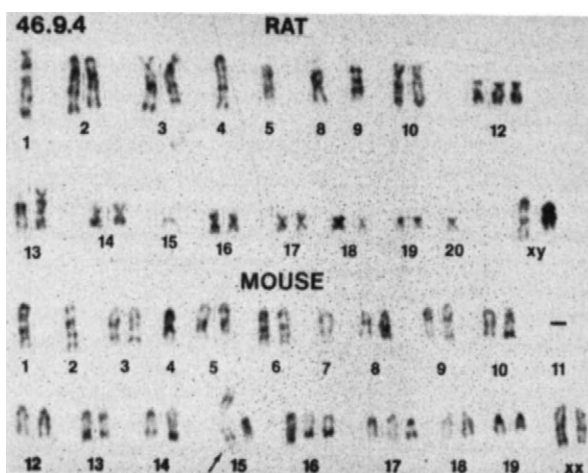


Fig. 1 Karyotype of a mouse $\times$ rat hybrid clone. Chromosomes were prepared using the ASG banding technique¹⁵. Four metaphases of 21.14 and 3 of 46.9 prepared on day 155 after fusion were classified according to Nesbitt and Franke¹⁶ for mouse and according to Dev *et al.*¹⁷ for rat chromosomes. All metaphases lacked mouse chromosome 11. The arrow indicates the isochromosome 15 which is the product of a robertsonian translocation of two mouse chromosomes 15 and which is a marker present in many CTL lines, including B6.1.SF.1⁴.

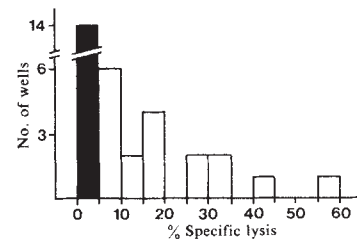


Fig. 2 Cytolytic activity of W/Fu(C58NT)D $\times$ B6.1.SF.1 hybrids selected in normal or CS-supplemented medium. W/Fu(C58NT)D was grown for two passages in medium containing thioguanine and ouabain to eliminate any revertants. 10^7 cells of either parent were washed, mixed and fused with 45% of polyethylene glycol in a procedure essentially identical to that described by Galfré *et al.*¹⁸ The cells were plated out at a density of 2×10^4 per cell type per well per 100 μ l in flat-bottom microtitre plates (96 wells, Nunc) in MLC medium¹⁹ or in MLC medium supplemented with 25% CS. 100 μ l of the same medium containing 10^{-4} M hypoxanthine, 5×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine, 2×10^{-3} ouabain and 2×10^{-5} M deoxycytidine were added to each well the next day. (This medium selects for cells in which the co-dominant ouabain resistance marker of W/Fu(C58NT)D and the wild-type hypoxanthine phosphoribosyl transferase gene of B6.1.SF.1 are both present; deoxycytidine is added to avoid any cytostatic effects of thymidine.) Thirteen days after fusion, cells from individual wells were detached by vigorous pipetting with a micropipette (Gilson) and transferred to round-bottom 96-well microtitre plates (Greiner). 10^4 51 Cr-labelled S194 target cells and Con A ($10 \mu\text{g ml}^{-1}$ final concentration) were added in 100 μ l of MLC medium. After 4 h of incubation at 37 $^{\circ}\text{C}$, specific isotope release was determined according to Brunner *et al.*²⁰. Spontaneous release was 7%. Hatched bars, wells containing MLC-medium; open bars, wells containing CS-supplemented MLC-medium.

Hybrids from this fusion, called PC60, grew in about half of the wells in either condition, whereas no growing cells were observed in a mock fusion without polyethylene glycol. Expression of antigens specific for B6.1.SF.1 (Thy 1.2) and W/Fu(C58NT)D (LC-antigen, Thy-1.1, AgB and OX19: refs 6–8 and M. Dallman and A. N. Barclay, unpublished) as determined by an indirect binding assay with radiolabelled anti-immunoglobulin antibodies, and the presence of both rat and mouse chromosomes in the karyotypes (Fig. 1) confirmed that the isolated cells were hybrids. Chromosome preparations prepared on days 88 and 155 after fusion from hybrids 41, 46 and 21 showed that the hybrids were chromosomally stable during this period. All clones retained a total of 68 to 66 chromosomes whereas the sum of the parental chromosomes is 79. Karyotypes of 46.9 and 21.14 at day 155 showed mainly loss of telocentric rat chromosomes and of both copies of mouse chromosome 11 (Fig. 1).

All hybrids selected in CS had significant lytic activity (Fig. 2). From two, 41 and 46, we obtained clones which were observed for 10 weeks after cloning and stably expressed lytic activity comparable with the parental CTL line (Fig. 3a). Clones of hybrid 46 but not of 41 were able to lyse S194 targets in the absence of Con A (Fig. 3a). All of the hybrids selected in CS showed, when cultured without CS, a transient decrease of their growth rate, but resumed rapid growth within a few days. Thus, we did not obtain stable, TCGF-dependent hybrids from this fusion.

None of 29 independent hybrids selected without CS had detectable cytolytic activity when first isolated (Fig. 2). However, on culture in CS for 3 days, four out of five became cytolytic. Two hybrids, 21 and 32, were cloned by micromanipulation in CS-supplemented medium. Clones 21.14 and 32.7 had, in the presence of Con A, activities close to the one of B6.1.SF.1 and were chosen for further studies (Fig. 3b). On culture in medium without CS (normal medium) these clones completely lost their activity, but could be induced again by re-exposure to CS (Fig. 4a). We consistently observed induction by CS of strong cytolytic activity with both clones in over 40

experiments. Induction and disappearance of cytolytic activity are complete within 48 to 72 h (Fig. 4a,b). Switch from CS-supplemented to normal medium or vice versa has no significant effect on the growth rate (Fig. 4c). No cytolytic activity was detected in W/Fu(C58NT)D cells after 3 days of culture in CS.

The data suggest a reversible induction phenomenon. However, cytolytic activity may be acquired by a terminal, irreversible differentiation process and it is possible that differentiated cells either cannot divide any further or become dependent on a growth factor in CS. In either case, the disappearance of cytolytic activity after removal of CS would reflect dilution of cytolytic cells by dividing cells which have not differentiated. If CS induces terminal differentiation and if differentiated cells cannot divide any further, one would expect that CS decreases the growth rate. However, there is no detectable effect of CS on the growth rate, over either short (Fig. 4c) or prolonged (30 day) periods. If differentiated cells become CS dependent, one would expect the cells, when taken out of CS, to resume exponential growth only after a lag period. This is not observed (Fig. 4c). Thus it appears unlikely that a large

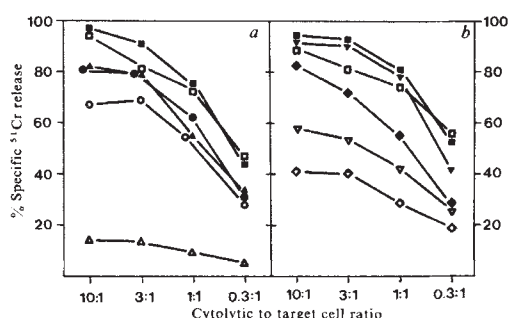


Fig. 3 Lectin dependence of cytolytic activity of rat $\times$ mouse hybrids. The cytolytic activity of hybrids selected in CS (a) or without CS (b) was tested in a 4-h (a) or 6-h (b) ^{51}Cr -release assay on 10^4 S194 targets in the presence (closed symbols) or absence (open symbols) of Con A ($10 \mu\text{g ml}^{-1}$ final dilution). The hybrids selected in the absence of CS had been grown in CS before the assay. $\blacksquare$, $\square$, B6.1.SF.1; hybrids; $\blacktriangle$, $\triangle$, 41.14; $\bullet$, $\circ$, 46.9; $\blacklozenge$, $\lozenge$, 21.14; $\blacktriangledown$, $\triangledown$, 32.7; a: 75 days after fusion; b, 112 days after fusion.

fraction of cells undergo irreversible differentiation. To rule out the differentiation model, it will be necessary to isolate single cytolytically active cells and to test whether they are able to proliferate in the absence of CS.

The inducing factor in CS is not ConA itself. It is precipitated by 80% but not by 40% ammonium sulphate. The inducing factor is retained in dialysis tubes with an average pore size of 15–20 Å and is stable when frozen at -20°C . Not only rat, but also mouse and human supernatants (SN) contain inducing activity. A supernatant obtained from a subline of the EL-4 mouse T lymphoma⁹ which has the same TCGF-titre as CS, fails to induce cytolytic activity even at a final concentration of 50% whereas CS induces significant cytolysis at a final concentration of 6% (Fig. 5a). As the EL-4 SN is not inhibitory when mixed with CS (Fig. 5a) and has no significant effect on cell proliferation (Fig. 5b), it seems either that TCGF is not involved in or not sufficient for the induction of cytolytic activity. Culture of B6.1.SF.1 in medium supplemented with EL-4 SN instead of CS for 30 days did not result in a significant decrease of cytolytic activity. Interferon has recently been claimed to be involved in the generation of CTL activity in mixed leukocyte culture¹⁰. We cannot induce cytolytic activity in the PC60 hybrids with up to 520 U per ml of type II interferon¹¹ produced by a murine T lymphocyte clone.

Fusion of B6.1.SF.1 with a rat T lymphoma has produced a class of cytolytic hybrids which do not depend on TCGF for growth and which therefore differ from those derived from intraspecific crosses between B6.1.SF.1 or other murine CTL

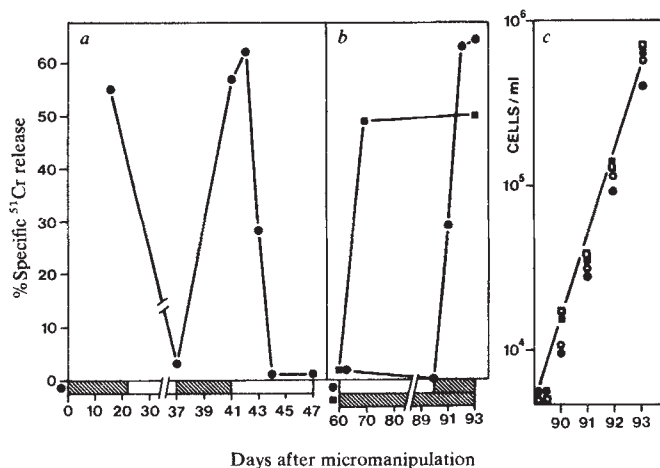


Fig. 4 Kinetics of induction and disappearance of cytolytic activity and of growth of hybrid 21.14. A colony was derived from a single cell of hybrid 21 micromanipulated on day 96 after fusion into a flat-bottom microtitre well containing 10^4 irradiated (2,000 R) mouse peritoneal macrophages in 200 μl of MLC medium supplemented with 25% CS. After the isolation the cells were further cultured in Petri dishes (Nunc) according to various schemes ($\bullet$, $\blacksquare$) in the medium indicated at the bottom: $\square$, MLC-medium; $\blacksquare$, MLC-medium supplemented with 25% CS. Cytolytic activity was measured in 6-h ^{51}Cr -release assays on S194 targets in the presence of $10 \mu\text{g ml}^{-1}$ Con A. Plots a and b represent specific ^{51}Cr release at a 10:1 cytolytic:target cell ratio. Cell growth (c) was measured by placing 5×10^3 cells per well (24 well, Costar) per ml of MLC-medium ($\circ$, $\square$) or MLC-medium supplemented with 25% CS ($\bullet$, $\blacksquare$). Before the assay cells had been maintained in medium with ($\square$, $\blacksquare$) or without ($\circ$, $\bullet$) CS as indicated in the bottom bar of Fig. 4. Cells were counted in a Coulter counter.

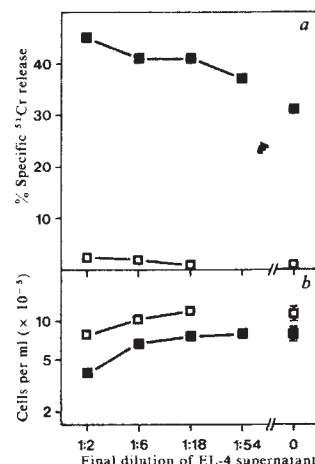


Fig. 5 Inducing capacity of EL-4 supernatant. EL-4 SN was produced by culturing EL-4 T lymphoma⁹ cells for 24 h in phorbol-myristate-acetate (10 ng ml^{-1} final dilution), CS by culturing rat spleen cells of outbred OFA rats for 48 h in the presence of Con A ($10 \mu\text{g ml}^{-1}$ final dilution). The supernatants were kept frozen in small aliquots and freshly thawed for each experiment. They contained equal concentrations of TCGF as determined by titrating their growth promoting activity on a TCGF-dependent CTL line (data not shown). Titration of the capacity of CS to induce cytolytic activity shows a plateau until a dilution of 1:8 (data not shown). 2×10^4 21.14 cells were cultured for 72 h in medium supplemented either with a suboptimal (1:16) dilution of CS plus various dilutions of EL-4 SN ($\blacksquare$) or in various dilution of EL-4 SN alone ($\square$). After 3 days of culture, cells were counted (b), and the cytolytic activity per cell was measured in a 6-h ^{51}C -release assay on S194 targets in the presence of $10 \mu\text{g ml}^{-1}$ Con A (a). Data represent lysis at a 10:1 cytolytic:target cell ratio.

lines and AKR thymomas^{1,2}. The TCGF-independent hybrids from the latter crosses could not be induced to become cytolytic by growth in CS. We also fused the W/Fu(C58NT)D line to another murine CTL line which depends both on TCGF and on feeder cells for growth, and we obtained the same type of inducible hybrids (A. S. and A. Glasebrook, unpublished). Preliminary analysis of these hybrids suggests that they express the same specificity as the parental CTLs. There are many possible reasons for the differences between the W/Fu(C58NT)D hybrids and those derived from the AKR T lymphomas. The most attractive is that these lines belong to different stages within the T lymphocyte differentiation lineage. *In vitro* generation of CTLs in mixed leukocyte culture seems to involve not only clonal expansion of CTLs but also a proliferation-independent transition of CTL precursors from an inactive to a cytolytic state¹². Recently, soluble factors other than

TCGF have been claimed to be involved in the induction of CTL activity in such systems (refs 13, 14, 21 and O. Kanagawa, manuscript in preparation). If the agent inducing cytolytic activity in the PC60 rat × mouse hybrids is related to these or other factors with similar properties, these hybrids will provide a tool for investigating the genetic control and the molecular mechanisms of the *in vitro* generation of cytolytic T lymphocytes.

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Reconstitution of antigen-specific suppressor activity with translation products of mRNA

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It has been well established that antigen-specific suppression of antibody responses can be mediated by soluble factors derived from suppressor T cells¹. However the chemical and functional properties of antigen-specific suppressor factors (TsF) are still controversial. We have recently described an antigen-specific suppressor T-cell factor composed of two distinct molecules. One is encoded by the gene located in the I-J subregion of the mouse H-2 complex on the 17th chromosome^{2,3}, and the other which possesses the antigen-binding moiety^{2,3} and the constant region determinant (Ct), is the product of the Ct gene linked to the immunoglobulin heavy chain (*Igh*) gene cluster on the 12th chromosome^{4,5}. The association of these two molecules is essential for the expression of the TsF activity^{2,3}. However, Fresno *et al.*⁶ have shown that the TsF specific for sheep red blood cells (SRBC) is composed of a single polypeptide chain carrying the antigen-binding moiety. Kapp *et al.*⁷ have described another TsF with the antigen (GAT)-binding moiety and the I-J determinants on the same molecule. We describe here the identification of messenger RNAs (mRNAs) coding for the Ct-bearing molecules with antigen-binding moiety and the I-J encoded molecules in mRNA fractions derived from the T suppressor cell hybridoma by translation in *Xenopus laevis* oocytes. Furthermore, the mixture of the translation products of these two separate mRNA fractions was shown to reconstitute the antigen-specific TsF activity.

The poly(A)-containing RNA was isolated from the suppressor T-cell hybridoma (34S-704)⁸ produced by the fusion of a thymoma, BW5147, and suppressor T cells of C57BL/6 mice, which is specific for keyhole limpet haemocyanin (KLH). The mRNAs were fractionated by sucrose gradient centrifugation and an aliquot of each fraction was injected into *X. laevis* oocytes.

In order to detect the minute amounts of the Ct-bearing and I-J-bearing molecules in the translation products of fractionated mRNA, we used a sensitive cytotoxicity inhibition assay with the suppressor T-cell hybridoma as a target. The translation products were tested for their ability to inhibit the cytotoxic activity of two monoclonal antibodies; F4 which was anti-I-J^b (B10.A(5R) anti-B10.A(3R)) and 7C5 anti-Ct^b which is specific for the constant region determinant (Ct) on the antigen-binding molecule of the TsF (BALB/c anti-CB-20).

The cytotoxicity of the monoclonal anti-I-J^b antibody (F4) was specifically inhibited by the addition of the translation products of 11S mRNA (Fig. 1). No other mRNA-directed

Table 1 Antigen-specificity of the TsF reconstituted by products translated from fractionated mRNAs

Translation products (μl ml ⁻¹)		Anti-DNP IgG PFC to	
11S mRNA	13S mRNA	DNP-KLH	DNP-OVA
0	0	1,730 ± 290	770 ± 90
0.15	0.15	1,650 ± 200	740 ± 60
0.30	0.30	1,280 ± 300	800 ± 80
0.60	0.60	600 ± 500	790 ± 120
1.25	1.25	90 ± 60	820 ± 100
2.50	2.50	30 ± 30	780 ± 80

The activity of the translation products of the fractionated mRNAs was tested in the *in vitro* secondary antibody response as described elsewhere¹². In brief, 8 × 10⁵ spleen cells from C57BL/6 mice primed 6 weeks previously with 100 μg DNP-KLH or DNP-OVA and 1 × 10⁹ *Bordetella pertussis* vaccine were cultured in a 96-well flat-bottomed plate (Falcon 3042 Falcon Labware, Oxford, California) with 200 μl of RPMI 1640 medium (Gibco) enriched with 10% fetal calf serum in the presence of 0.1 μg per ml DNP-KLH or DNP-OVA and 2 × 10⁻⁵ M 2-mercaptoethanol. The different doses of the mixture of the translation products of 11S and 13S mRNAs were added to the culture at the start. The translation products used are a different batch from those in Figs 1 and 2. The culture was maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C. Five days later, the numbers of anti-DNP-IgG PFC were counted in a Cunningham chamber by using DNP-coupled SRBC. Results are expressed as arithmetic means of the numbers of anti-DNP IgG PFC of six cultures ± s.d.

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translation products nor the oocyte extracts showed significant inhibition of the cytotoxicity. However, the translation products of 13S and 18.5S mRNA contained the Ct molecules, because they inhibited the cytotoxic activity of the monoclonal BALB/c anti-CB-20 antibody (7C5). Furthermore, the 12S mRNA-directed products (fraction 12) shown in Fig. 1 also appeared to contain the Ct molecule. However, the inhibitory effect was not always obtained when different batches of 12S mRNA products were used, perhaps because of the conditions of the fractionation. Therefore, 13S mRNA seems to encode the Ct molecules.

Fractionation by SDS-polyacrylamide gel electrophoresis was performed on the 11S, 13S and 18.5S mRNA-directed translation products to determine their molecular weights (M_r s). The materials from the sliced gels were eluted by electrophoresis. The eluates were then added to the cytotoxic assay system using

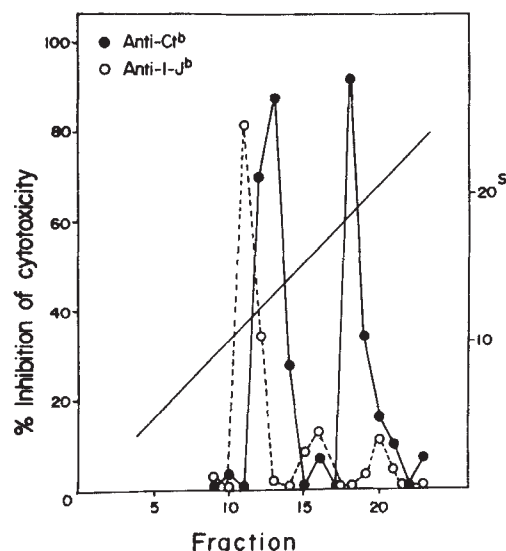


Fig. 1 Analyses of translation products of hybridoma-derived mRNA fractionated by sucrose density gradient centrifugation. The Ts cell hybridomas (34S-704) were grown subcutaneously in (AKR $\times$ C57BL/6) F_1 mice. Cytoplasmic RNA was extracted from 50 g of the hybridoma cells as described previously¹³ except that the homogenizing buffer was replaced by 0.1 M Tris-HCl (pH 9.0) containing 0.25 M sucrose, 0.1 M NaCl and 5 U per ml RNase inhibitor¹³ or rat liver. Poly(A)-containing RNA was enriched by two successive applications onto an oligo(dT) cellulose column and further fractionated by sucrose density gradient centrifugation as described elsewhere¹⁵. An aliquot of the fractionated mRNA was injected into *X. laevis* oocytes. Ten oocytes injected with the same fraction of mRNA were incubated in 100 μ l of a sterile modified Barth's medium¹⁶ at 19–21 $^{\circ}$ C for 36 h. The translation products were prepared by centrifugation of the oocyte homogenates at 100,000g for 1 h. Translation products of each mRNA fraction were tested for their ability to inhibit the cytotoxic activity of the monoclonal antibody against the I-J or the Ct molecules. The target cell used was the I-J⁺, Ct⁺ Ts hybridoma (F8C28). 10^4 target cells and 10 μ l of ascites containing monoclonal antibodies from the F4 clone (anti-I-J^b)¹⁷ at a 1:2,000 dilution or those from the 7C5 clone (anti-Ct^b)¹² at a 1:320,000 dilution, that gave the end point of maximum cytotoxicity, were mixed with 10 μ l of the translation products or the oocyte extracts as an inhibitor. The mixture was incubated for 30 min on ice in a V bottomed microtitre plate (Dynatech). The cells were washed, pelleted and resuspended in 20 μ l of rabbit complement (C) followed by further incubation for 30 min at 37 $^{\circ}$ C. Dead and viable cells were counted under the light microscope by a trypan blue dye exclusion. The per cent cytotoxicity and per cent inhibition of the cytotoxicity were calculated according to the following formulae:

$$\% \text{ Cytotoxicity} = 100 \times \frac{\% \text{ dead exp.} - \% \text{ dead C cont.}}{100 - \% \text{ dead C cont.}}$$

$$\% \text{ Inhibition of the cytotoxicity} = 100 \times \left(1 - \frac{\% \text{ cytotoxicity exp.}}{\% \text{ cytotoxicity max.}} \right)$$

monoclonal anti-I-J^b (F4) or anti-Ct^b (7C5) antibodies. The molecule with maximal inhibitory activity on the cytotoxicity of the monoclonal anti-I-J^b had a molecular weight of 25,000–27,000 when the translation products of 11S mRNA were fractionated and assayed. Similarly, the Ct-bearing molecule in the 13S mRNA products was shown to have a M_r of 29,000–34,000. The 18.5S mRNA translation products appear to contain the Ct molecule of 45,000–62,000 M_r . It is clear, therefore, that the I-J molecule is encoded by 11S mRNA and has a M_r of 25,000–27,000. It was unexpected that the Ct-bearing molecules are coded for by two distinct types of mRNAs. Inasmuch as 13S and 18.5S mRNAs direct synthesis of the Ct-bearing molecules with different sizes, 18.5S mRNA is unlikely to be an aggregate of 13S mRNA. The small and large Ct-bearing molecules might represent the secreted and membrane-bound forms, respectively, of the antigen-binding molecules of suppressor T cells, like two forms of immunoglobulin heavy chains^{9–11}.

The next experiments were carried out to investigate the functional activities of the translation products. Our previous studies^{2,3} have shown that the mixture of the dissociated I-J encoded products and the KLH-binding molecules, neither of which could exert any suppressor activity by itself, reconstitutes the KLH-specific TsF activity. Therefore, the translation products of 11S mRNA coding for the I-J molecule were mixed with the product of each fractionated mRNA, and the mixture was tested for suppressor activity in the *in vitro* antibody response of C57BL/6 spleen cells primed with 2,4-dinitrophenylated-KLH (DNP-KLH).

No significant effect on the antibody response was observed by the addition of 10–20 μ l ml⁻¹ of oocyte extracts or the translation products of each fractionated mRNA by themselves. However, when the translation products of either 13S or 18.5S mRNA were mixed with the 11S mRNA products, the mixture successfully reconstituted the TsF activity (Table 1). Translation products of fractionated mRNAs other than 13S and 18.5S mRNAs could not reconstitute the TsF activity even in the presence of 11S mRNA products (data not shown). These results suggest that both forms of Ct-bearing molecules can reconstitute the active form of TsF when mixed with the *in vitro* translated I-J molecules.

The suppression of the antibody response mediated by the mixture of the translation products of 11S and 13S mRNAs was shown to be KLH specific, because the materials suppressed the anti-DNP IgG plaque-forming cell (PFC)

Table 2 Reconstitution of TsF activity with the I-J and the Ct molecules translated by fractionated mRNAs

mRNA-products (μ l ml ⁻¹) applied with the column				
Anti-I-J ^b (11S mRNA)	KLH (13S mRNA)	Anti-Ct ^b (13S mRNA)	Anti-DNP IgG Eluate	PFC per culture Effluent
—	—	—	1,430 $\pm$ 290	1,430 $\pm$ 290
2.5	—	—	1,350 $\pm$ 300	1,490 $\pm$ 250
—	2.5	—	1,410 $\pm$ 280	1,430 $\pm$ 110
—	—	2.5	1,400 $\pm$ 180	1,360 $\pm$ 210
1.25	1.25	—	140 $\pm$ 180	1,650 $\pm$ 180
1.25	—	1.25	270 $\pm$ 120	1,390 $\pm$ 150
—	1.25	1.25	1,450 $\pm$ 300	1,510 $\pm$ 300

As the 11S or 13S mRNA was shown to encode the I-J molecules or the KLH-binding molecules with the constant region determinants of the T-cell antigen receptors, respectively, the translation products of the 13S mRNA were applied to the immunoabsorbent columns of the KLH (5 mg KLH per 1 ml Sepharose beads) or the monoclonal alloantibodies specific for the constant region determinant (Ct) of the T-cell antigen receptor (5 mg of monoclonal BALB/c anti-CB-20 antibodies per ml of beads). Similarly, the products of 11S mRNA were absorbed with the anti-I-J^b column (1 ml of γ -globulin fraction of B10.A(5R) anti-B.10A(3R) per 1 ml beads). The translation products used are the same as those in Table 1. The materials absorbed on the columns were eluted with 0.175 M glycine HCl buffer, pH 3.2. The eluates were instantly neutralized with 0.4 M sodium bicarbonate and dialysed extensively with 0.01 M sodium phosphate-buffered saline, pH 7.2. They were then concentrated by a vacuum pressure. The suppressor activity in the eluate or the effluent from a single column or in the mixture of the half volume of the materials from the two independent columns was tested in the *in vitro* secondary antibody response as described in Table 1 legend. Results are expressed as arithmetic mean numbers of the anti-DNP IgG PFC of six cultures $\pm$ s.d.

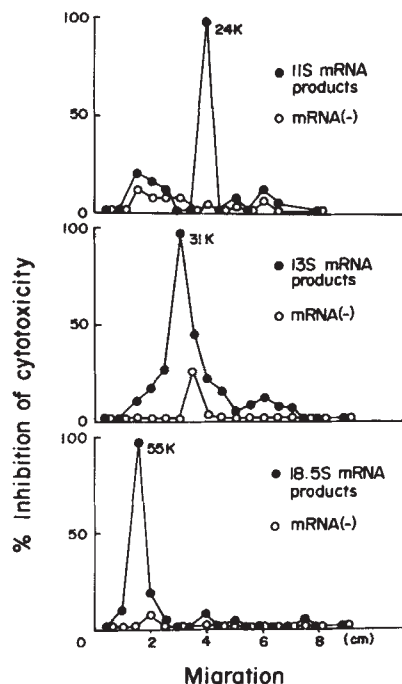


Fig. 2 Molecular weights of the I-J and the Ct molecules translated in *X. laevis* oocytes. The translation products of 11S, 13S and 18.5S mRNA were electrophoresed in SDS-polyacrylamide gels (20%) with the discontinuous buffer system¹⁸. The gels were frozen and sliced to 5 mm width and the translation products were eluted by electrophoresis. The eluates were assayed for their ability to block the cytotoxicity of the monoclonal antibodies as described in Fig. 1. For each experiment oocyte extracts without mRNA were run as a control. Molecular weight markers used are bovine albumin (66K), ovalbumin (45K), hog pepsin (34.7K), bovine trypsinogen (24K), egg white lysozyme (14.3K) and horse cytochrome c (12.4K).

responses to DNP-KLH but not to DNP-ovalbumin (DNP-OVA) (Table 1). The magnitude of the antigen-specific suppression was dependent on doses of the translation products added. These results support our previous data⁸ showing that the TsF obtained from KLH-primed spleen cell extracts or hybridoma extracts, suppressed the antibody response in an antigen-specific fashion.

Although the antigen-specific TsF activity is reconstituted by the combination of the translation products of 11S mRNA and either one of 13S or 18.5S mRNA, it has not been demonstrated that the I-J and the KLH-binding molecules in the translation products of 11S and 13S mRNAs are, in fact, responsible for the reconstitution of the antigen-specific TsF function. To show this, the 11S or 13S mRNA products were applied to columns composed of KLH, anti-I-J or anti-Ct (7C5). The adsorbed materials were then eluted with 0.175 M glycine HCl buffer, pH 3.2. The eluate, the effluent or the mixture of the materials from the columns was tested for suppressor activity on the *in vitro* secondary antibody responses. No suppressor activity was observed in the acid eluate of translation products from either column. However, the mixture of the eluate from the anti-I-J^b column and that from either the KLH or the anti-Ct (7C5) column demonstrated maximum suppression (Table 2). The effluents had no activity. From these results, it appears that the 11S mRNA encodes the I-J molecule and the 13S mRNA codes for the KLH-binding molecule with constant region determinants (Ct) of the T-cell antigen receptor, and that the association of the translation products of the I-J and the Ct-bearing molecules reconstitutes the active form of the KLH-specific suppressor T-cell factor. The polypeptide chain with the constant region determinants defined by the anti-Ct (7C5) binds to the antigen by itself, so that it could act as the element responsible for the antigen specificity of the TsF. The

I-J molecule on TsF might act as a functional molecule to convey suppressor activity. However, this does not imply that the I-J molecules themselves exert the suppressor function, because alone they did not show any suppressor activity (Table 2).

It has recently been shown that the antigen-specific augmenting T-cell factors (TaF) that enhance antibody responses are composed of I-A encoded products and Ct molecules distinct from those for the TsF⁴. It is therefore important to investigate the relationship between the role of the MHC gene products and the polymorphism of the Ct molecules in the functional expression of T-cell factors. Experiments to answer these questions are now in progress.

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Constant region determinants on the antigen-binding chain of the suppressor T-cell factor

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Antigen recognition units on T cells, including isolated antigen receptors and antigen-specific T-cell factors, have been found to have antigen-binding moieties like conventional immunoglobulins and to carry the determinants analogous to the variable (V) region of the immunoglobulin¹⁻⁵. However, the immunoglobulin heavy chain constant region (Igh-C) determinants have not been demonstrated on T-cell receptor molecules⁶⁻⁹. If they possess unique constant region determinants, they must therefore be entirely distinct from the Igh-C. By production of an antiserum between Igh allotype congenic mice Owen *et al.* suggest that a T-cell constant region determinant (Tsu⁴) is encoded by a gene located near the Igh-C genes on the 12th chromosome^{10,11}. In addition, we have also demonstrated that a BALB/c anti-CB-20 antiserum detects at least two distinct allotypic determinants, either of which is expressed on the antigen-specific suppressor or augmenting T-cell factor¹². We now demonstrate that the new allotypic determinants detected by the BALB/c anti-CB-20 antiserum are expressed on the constant region of the antigen-binding molecule of an antigen-specific suppressor T-cell factor (TsF), and that this molecule is encoded by genes located on the right side of the *Igh-V* gene locus on the 12th chromosome.

Table 1 Successful absorption of the antigen-specific suppressor T-cell factors from *Igh-1^b* mice with anti-CB-20

TsF absorbed with	Materials added	IgG anti-DNP PFC per culture KLH-TsF obtained from mice				
		C57BL/6 (<i>H-2^b, Igh-1^b</i>)	CWB (<i>H-2^b, Igh-1^b</i>)	C3H.SW (<i>H-2^b, Igh-1ⁱ</i>)	BALB/c (<i>H-2^d, Igh-1^a</i>)	BAB-14 (<i>H-2^d, Igh-1^b</i>)
—	—	10,500 ± 2,050	19,750 ± 3,250	9,900 ± 1,200	11,750 ± 400	8,600 ± 700
None	Unabsorbed TsF	500 ± 300	6,450 ± 1,200	900 ± 550	700 ± 600	850 ± 750
Anti-CB-20	Effluent	9,750 ± 1,650	17,250 ± 1,650	450 ± 350	650 ± 400	10,050 ± 1,000
Anti-CB-20	Euate	750 ± 450	5,650 ± 850	10,550 ± 900	11,450 ± 850	900 ± 550

The TsF activity was tested in an *in vitro* secondary antibody response as described elsewhere⁶. Briefly, 4×10^6 spleen cells from C57BL/6 mice, primed 6 weeks previously with 100 µg DNP₇₇₀-KLH and 1×10^9 *B. pertussis* vaccine, were cultured in the Mishell-Dutton system, using plastic plates (Falcon Labware, 3008), with 1 ml of RPMI 1640 (Gibco) enriched with 10% fetal calf serum in the presence of 0.1 µg DNP₇₇₀-KLH and 2×10^{-5} M 2-mercaptoethanol. The cultures were maintained in an humidified atmosphere of 5% CO₂ in air at 37 °C. Five days later, the numbers of anti-DNP IgG plaque forming cells (PFC) were assayed by using DNP-coupled erythrocytes. For the preparation of the suppressor T cell factor (TsF), spleen cells primed twice with 200 µg of KLH at 2-week intervals were disrupted by freezing and thawing. The cell-free extracts were obtained by ultracentrifugation of the extracted materials at 40,000g for 1 h. The cell-free extracts equivalent to 1×10^7 spleen cells from C57BL/6, C3H.SW, CWB, BALB/c or BAB-14 mice were absorbed to, and incubated with, the immunoadsorbent column of BALB/c anti-CB-20 (1 ml γ-globulin fraction of antiserum per 1 ml beads) for 1 h at 4 °C. The effluent was collected by passing it through the column. After washing with RPMI 1640 medium, the absorbed materials were eluted from the column with 2 ml of 0.175 M glycine HCl buffer pH 3.2, at 4 °C. The eluted materials were instantly neutralized with 0.4 M sodium bicarbonate and dialysed with 0.01 M sodium phosphate-buffered saline, pH 7.2. They were then concentrated by vacuum pressure. The suppressor activity of the effluent and the eluate from the column was tested in a culture of syngeneic, or H-2 identical recipients, primed with DNP-KLH at the start of cultivation. The TsF from C3H.SW and CWB mice was added to the culture of C57BL/6 mice primed with DNP-KLH. Similarly, the activity of the BAB/14 TsF was assayed in the BALB/c spleen cell culture, since the identity of phenotypes of I-J subregion genes between the TsF and the responder strain is essential for the expression of the TsF activity¹⁶. Results are expressed as mean numbers of anti-DNP IgG PFC of five cultures ± s.d.

Our previous studies showed that the alloantiserum specific for T cells could be successfully raised in BALB/c mice hyper-immunized with concanavalin A (Con A)-stimulated *Igh* allotype congenic CB-20 spleen cells, and that the BALB/c anti-CB-20 antiserum reacts with the allotypic determinants expressed only on some T cells but not on B cells of the *Igh-1^b* mice, as the anti-CB-20 could eliminate ~20% of thymocytes from the *Igh-1^b* mice (C57BL/6, SJL, CWB and BAB-14) but not those from mice having other *Igh-1* allotypes, that is, *Igh-1^a* (SJA and BALB/c), *Igh-1^d* (AKR) and *Igh-1ⁱ* (C3H and C3H.SW)¹². It could also absorb the TsF¹².

The genetic specificity of the BALB/c anti-CB-20 antiserum was examined by using the TsF derived from various strains of mice having different *Igh* allotypes. Suppressor T-cell factors specific for keyhole limpet haemocyanin (KLH) were prepared by a freeze and thaw treatment of spleen cells from C57BL/6 (*H-2^b, Igh-1^b*), BALB/c (*H-2^d, Igh-1^a*), BAB-14 (*H-2^d, Igh-1^b*), C3H.SW (*H-2^b, Igh-1ⁱ*) and CWB (*H-2^b, Igh-1^b*) mice immunized twice with 200 µg of KLH at 2 week intervals as described elsewhere⁶. These KLH-TsF were further absorbed with the anti-CB-20 column. The absorbed materials were then eluted with 0.175 M glycine-HCl buffer, pH 3.2. The effluent and the eluate from the columns were tested for activity in the *in vitro* secondary antibody response of spleen cells of mice primed with DNP-KLH and *Bordetella pertussis* vaccine (BPV) as described previously⁶. The suppressor activity of the KLH-TsF derived from C57BL/6, BAB/14 and CWB (*Igh-1^b*) but not from BALB/c (*Igh-1^a*) and C3H.SW (*Igh-1ⁱ*) mice was absorbed to and eluted from the anti-CB-20 column (Table 1). Therefore, the anti-CB-20 antiserum recognized the allotypic determinants on the antigen-specific TsF of the *Igh-1^b* mice, suggesting that these allotypic determinants on the TsF are encoded by genes linked to the *Igh-1^b* genes. As the anti-CB-20 has been made by immunizations with unprimed Con A-stimulated spleen cells, it is unlikely that the antibodies are directed against the idiotype-like determinants of the TsF. Therefore, it seems that the allotypic determinants detected by the antiserum are expressed on the constant region rather than the variable region of the TsF.

The next experiments were designed to investigate this. First, we tried to show that the allotypic markers are present on the antigen-binding molecule of the TsF. Our previous results have shown that the factor in the extracted materials of a suppressor T-cell hybridoma is composed of two distinct gene products in noncovalent association, one encoded by genes in the I-J sub-region of the H-2 complex, and the other having the antigen-binding moiety¹³. The association of these two molecules has

been found to be necessary for the expression of the TsF activity¹⁴.

The KLH-specific suppressor factor derived from a T-cell hybridoma (34S-704) was absorbed with columns of the KLH, anti-I-J^b or anti-CB-20 antiserum. The effluents from the columns or a mixture of half volume of these two were added to the *in vitro* secondary culture system. The suppressor activity of the hybridoma-derived KLH-TsF was completely absorbed with these columns. However, a mixture of a half volume of the effluents from both the KLH and anti-I-J^b columns reconstituted strong suppressor activity (Table 2). The suppressor activity was also reconstituted, when the effluent from the anti-CB-20 column was mixed with that from the anti-I-J^b but not that from the KLH column. Therefore, the results in Table 2 indicate that the anti-CB-20 column absorbed the KLH-binding but not the I-J encoded molecule. A similar result was obtained with the KLH column, suggesting that the KLH-binding molecules of the TsF possess the allotypic determinants controlled by the *Igh*-linked genes on the 12th chromosome.

Table 2 The anti-CB-20 antiserum reacts with antigen binding but not I-J-bearing molecules of the TsF

Unabsorbed TsF	Materials added to the culture			IgG anti-DNP PFC per culture
	Effluent from the column composed of	KLH	Anti-I-J ^b Anti-CB-20	
—	—	—	—	1,170 ± 30
5×10^5	—	—	—	120 ± 30
—	10×10^5	—	—	1,240 ± 250
—	—	10×10^5	—	1,260 ± 90
—	—	—	10×10^5	1,170 ± 150
—	5×10^5	5×10^5	—	140 ± 60
—	—	5×10^5	5×10^5	90 ± 90
—	5×10^5	—	5×10^5	1,130 ± 230

The monoclonal KLH-TsF extracted from 3×10^6 hybridoma cells (34S-704) was absorbed by passing through the following immunoadsorbent columns: KLH (5 mg KLH per 1 ml beads), anti-I-J^b (B10.A(5R) anti-B10.A(3R); 1 ml γ-globulin fraction of antiserum per 1 ml beads), BALB/c anti-CB-20. The method for preparation of immunoadsorbents was described previously¹². The effluent from the column or the mixture of half volume of the two different effluents was added to the culture of spleen cells from the DNP-KLH-primed C57BL/6 as described in Table 1 legend. The numbers are those of the hybridoma cells used as the source of the TsF. Results are expressed as mean numbers of anti-DNP IgG PFC of five cultures ± s.d.

Table 3 Anti-CB-20 reacts with the constant region of the antigen-specific suppressor T-cell factor

Immuno-adsorbent column	Materials added	IgG anti-DNP PFC/culture	KLH-TsF	OVA-TsF
—	—	8,400 ± 1,640	400 ± 240	2,680 ± 410
None	Unabsorbed TsF			330 ± 130
Anti-CB-20	Effluent	7,760 ± 1,320		2,400 ± 440
Anti-CB-20	Eluate	600 ± 360		220 ± 110

The cell-free extracts equivalent to 1×10^7 spleen cells from C57BL/6 mice immunized intraperitoneally twice with 200 μ g of KLH or OVA at 2-week intervals were absorbed with the anti-CB-20 column as described in Table 1 legend. The suppressive activity of the effluent and eluate from the column was added to the *in vitro* secondary antibody response of DNP-KLH-primed or DNP-OVA-primed C57BL/6 spleen cells, respectively. Results are expressed as mean numbers of anti-DNP IgG PFC of five cultures $\pm$ s.d.

Second, the results shown in Table 3 suggest that the anti-CB-20 serum recognizes constant region determinants on the TsF. In this experiment, the conventional KLH-TsF and Ovalbumin-specific TsF (OVA-TsF) were obtained by the freezing and thawing of the spleen cells of C57BL/6 mice primed twice with 200 μ g KLH or OVA as described previously⁶. The KLH-TsF was absorbed with the anti-CB-20 column. The materials were also eluted with 0.175 M glycine-HCl buffer, pH 3.2. The suppressor activity of the KLH-TsF or the OVA-TsF in the effluent or the eluate was tested in the *in vitro* secondary antibody responses to DNP-KLH or DNP-OVA, respectively. The activities of both KLH-TsF and OVA-TsF were absorbed to and eluted from the same anti-CB-20 column. As the two suppressor factors should have distinct variable regions it seems that the allotypic determinants detected by the anti-CB-20 are the shared determinants on the antigen-binding molecules of the TsF with different antigen specificities. The same results were also obtained by our recent studies using monoclonal BALB/c anti-CB-20 antibody (7C5) specific for the TsF.

The results (Tables 2 and 3) suggest that the antigen-binding molecule of the TsF possesses variable and constant region domains and that the anti-CB-20 recognizes the determinants on the constant region domains of the antigen-binding molecules of TsF. Genes coding for the constant region determinants seem to be located on the right side of the *Igh-V* gene cluster on the 12th chromosome, because the anti-CB-20 could absorb the TsF from BAB-14 mice carrying the *Igh* constant (*Igh-C'*) genes from C57BL/Ka (*Igh-1^b*) mice and the *Igh-V* genes from BALB/c (*Igh-1^a*) mice¹⁵ (Table 1). However, it is possible that the anti-CB-20 recognizes allotypic markers on the variable region domains instead of the constant region domains of the antigen-binding molecule of TsF, if there are sets of *V* gene clusters for T-cell receptors on the right side of the *Igh-V* gene pools.

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Isolation of malignant and functional Ly 1⁺ T-cell clones from B-cell lymphomas

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Recent work has shown that established B-cell tumours, previously thought to proliferate independently of immune regulation, may activate, and be regulated by, helper or suppressor T-cell subpopulations^{1–7}. We now report a unique model of a murine sarcoma virus (MSV) induced B-cell lymphoma which is closely associated with a population of pre-malignant or malignant helper T cells. Proliferation of these T cells is not observed *in vivo* but is readily detected on *in vitro* cultivation. These results raise questions about immunological control and differentiation of malignant lymphomas *in vivo*.

Twenty 3-week-old C57BL/6(B6) mice (Jackson Laboratories) were injected intramuscularly (i.m.) with 6×10^3 focus-forming units of murine sarcoma-murine leukaemia virus (MSV). This virus was generated from tumour cell suspensions of MSV-induced rhabdomyosarcomas induced in young BALB/c By mice. The B6 mice were observed daily for the first 3 weeks following virus infection. Those mice which did not develop a palpable tumour at the site of inoculation after 21 days were eliminated from the experiment (3/20). The remaining mice were observed weekly for 2 or more years, after which time 15/17 developed mesenteric lymphadenopathy. Cell suspensions were prepared from an enlarged mesenteric lymph node and 20×10^6 cells injected intraperitoneally (i.p.) into 20 normal B6 mice. All those mice developed splenic and lymph node tumours 3 months later.

The transplantable tumour, called STC, produced progressive expansion of the splenic white pulp and of the paracortical and medullary cord areas of lymph nodes. The tumour infiltrates consisted mostly of small lymphocytic cells with varying degrees of plasmacytoid cytoplasmic differentiation (Fig. 1a). Frequent periodic acid Schiff (PAS) positive cytoplasmic and intranuclear inclusions were present. Ultrastructural studies showed abundant endoplasmic reticulum in the tumour cells (Fig. 1b) which were often distended, presumably by cytoplasmic immunoglobulin (Ig). Further studies using immunofluorescence techniques confirmed that the cells were cytoplasmic μ -chain positive and >80% of the cells in lymphocyte cell suspensions derived from these spleens had surface membrane IgM. These features of STC are identical to those of the plasmacytoid lymphocytic type of human malignant lymphoma of B-cell origin, according to the criteria of Lukes and Collins⁸.

To determine whether STC could be transferred by B cells, a single cell suspension was made from the spleen of a B6 mouse injected with STC. B cells were enriched from the cell suspension using an immunoabsorbent column technique⁹. They were then treated in two steps with a 1:10, then 1:20 dilution of heat-inactivated rabbit anti-mouse brain antiserum (BAT)¹⁰ and a 1:8 dilution of absorbed guinea pig complement to deplete contaminating T cells. After four washes in RPMI 1640 plus 10% fetal calf serum, an aliquot of cells was evaluated for surface immunoglobulin using a fluoresceinated rabbit anti-mouse F(ab')₂ reagent and 99% of the cells were found to be Ig⁺. 10×10^6 of these cells were then injected i.p. into normal B6 mice. The neoplasms which developed in these mice were

morphologically identical to those found in animals injected 2 yr previously with MSV or with STC. These results showed that the malignant lymphoma induced by MSV could be transferred by a population of highly enriched B cells, further depleted of contaminating T cells.

In an attempt to establish B-cell clones, cells were obtained from the mesenteric lymph node of a B6 mouse injected with MSV and cultured in RPMI 1640, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin-streptomycin and 1.2% HEPES buffer. The cells were plated at limiting dilutions (0.3 cells per well) in Falcon microtitre dishes on a syngeneic feeder layer. However, as seen in Fig. 2, the cells

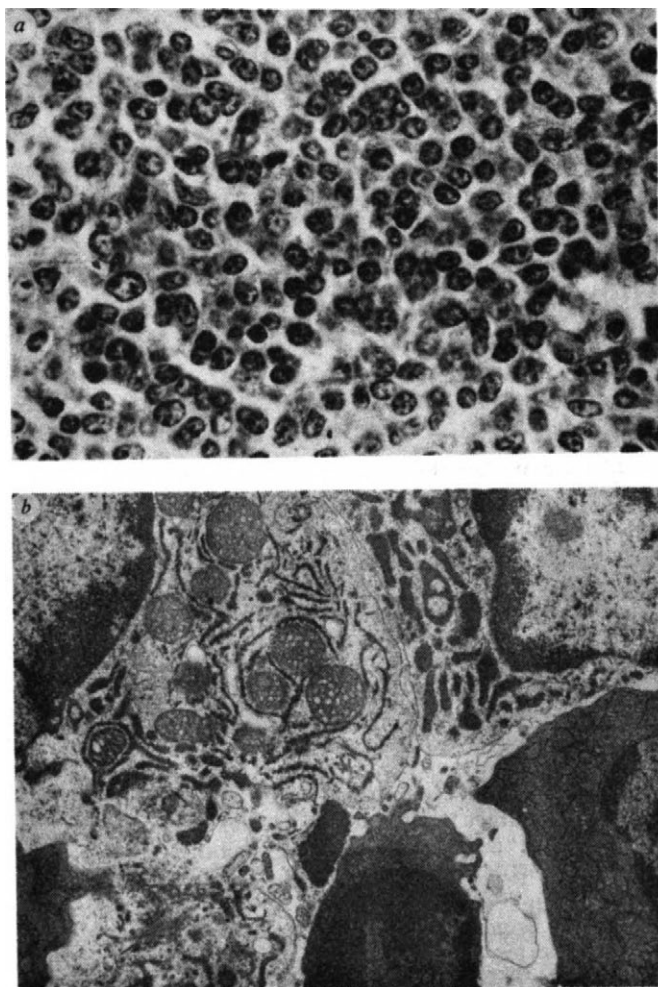


Fig. 1 *a*, Spleen cells derived from a C57BL/6 mouse injected with MSV-derived malignant lymphoid cells ($\times 290$). *b*, 0.2 g of tissue were fixed in phosphate-buffered saline plus 1.25% glutaraldehyde. The specimen was placed in 2.5% glutaraldehyde and embedded in Epon-Araldite. Sections were cut at 50–150 μm .

which proliferated as a result of the cloning procedure were not B cells but Ly 1⁺ T cells. One clone, designated clone A, reacted with monoclonal antibodies identifying the Ly 1 but not Ly 2 surface antigens. Ten additional clones have been established from the mesenteric lymph nodes of individual B6 mice injected with MSV-MuLV-M or STC. In each instance the phenotype of the cells was Thy 1.2⁺ (monoclonal antibody 30 H12, ref. 11), Ly 1⁺, Ly 2⁻.

Doses of clone A or similar T-cell clones were injected i.p. into normal B6 mice and as few as 1×10^6 cells killed syngeneic mice in 1 week. These tumours, however, were immunoblastic T-cell sarcomas, morphologically distinct from the plasmacytoid lymphocytic lymphomas induced by MSV, STC or B cells. So far, two clones have been injected into B6 mice at a dose of 1×10^6 cells per mouse. After 6 days, cells were obtained from the tumour and re-cloned. The cells which proliferated *in vitro*

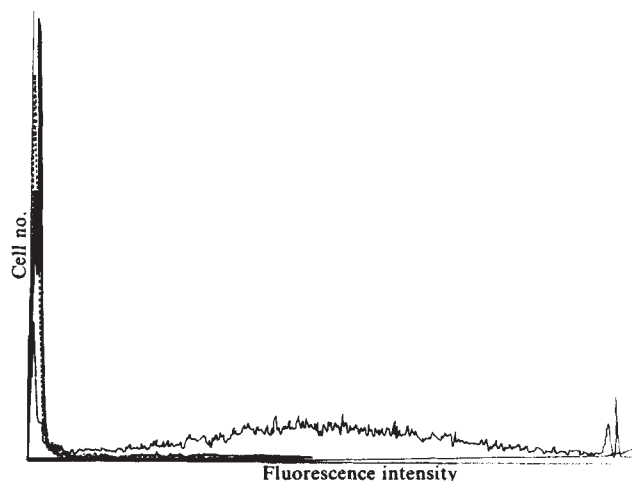


Fig. 2 Immunofluorescence profile of clone A incubated with rat monoclonal antibodies identifying the Ly 1 (—) or Ly 2 (---) differentiation antigens. (The rat cell lines were obtained from Dr N. Warner.) 1×10^6 clone A cells were incubated with 0.15 ml of a 1:50 dilution of rat IgG (0.8 mg per ml) (---), anti-Ly 1 (—), or anti-Ly 2 (---) at 4°C for 30 min, centrifuged and washed three times. 40,000 cells were then analysed on a FACS II (Beckton-Dickinson) and the intensity of fluorescence per cell recorded on a pulse height analyser.

retained the original phenotype, Thy 1.2⁺, Ly 1⁺, Ly 2⁻ (Fig. 3), showing that the clones, even on *in vivo* passage, were morphologically stable.

Experiments were then carried out to determine if the cloned malignant T cells were functional. Unseparated spleen or normal B cells were incubated with T cells or clone A cells in a reverse haemolytic plaque assay¹². The number of cells spontaneously secreting immunoglobulin was then ascertained. The three experiments show that the addition of clone A cells to either normal spleen cell populations or highly enriched B cells resulted in enhanced immunoglobulin secretion (Fig. 4): twice the number of plaque-forming cells were detected when the B cells were incubated with clone A (column O) rather than T cells (column M). These results demonstrated that this clone (and others tested similarly) retained at least one normal T-cell function, the ability to enhance Ig secretion by plasma cells in the absence of antigen.

The present study reports a new model of MSV-induced lymphomagenesis. MSV induced a malignant B-cell lymphoma in old B6 mice which was histologically stable on serial transplantation. By several criteria, the lymphoma is analogous to the plasmacytoid lymphocytic lymphoma of humans. However, the lymphoma transferred by enriched B cells was clearly different from the immunoblastic T-cell sarcoma induced by

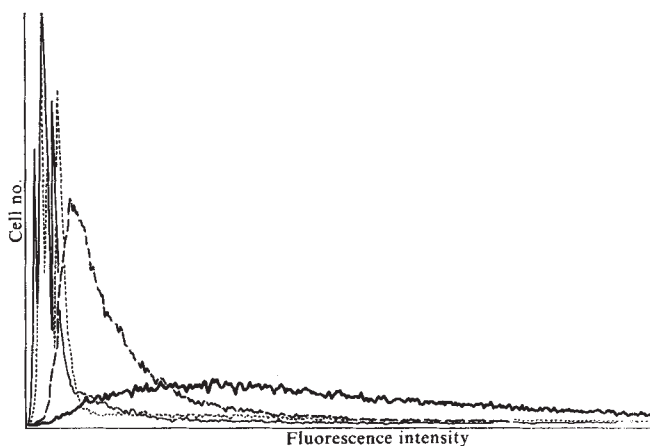


Fig. 3 Immunofluorescence profile of clone BB6S incubated with rat monoclonal antibodies identifying the Ly 1 (—), Ly 2 (.....) or 30 H12 (---) differentiation antigens. Normal rat serum was used as a control (---). All procedures are identical to those for Fig. 1.

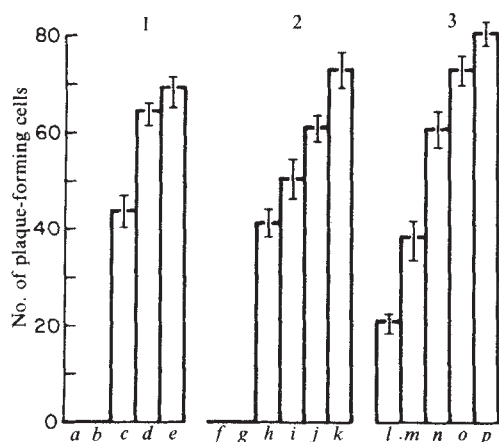


Fig. 4 Enhancement of spontaneous immunoglobulin secretion by T cells or aberrant Ly 1⁺ T-cell clones. 50 μ l of 10^6 lymphocytes per ml in serum-free RPMI 1640 and 50 μ l of a 15% solution of coated sheep red blood cells (SRBC) were pipetted into 10 $\times$ 75 glass test tubes containing 0.9 ml of an 0.8% solution of sea-plaque agarose (Marine Colloids) in Hank's balanced salt solution (HBSS; Gibco). SRBC were coated by treating the cells with chromium chloride at a dilution of 1 mg per ml in 0.85% saline. They were then incubated with R/M F(ab')₂ antisera at a concentration of 1 mg per ml in saline. The SRBC were washed three times, resuspended to a concentration of 15% in saline and used as indicated above. RPMI 1640 was aliquotted at 1 ml per plate. The plates were incubated for an additional hour, the developer decanted, and 1 ml guinea pig complement diluted 1:20 in RPMI 1640 was added. The incubation was continued for an additional hour and the plaques counted. (See ref. 12.) Student's *t*-test was carried out on all the results and *t*-values with their significance (*P*) are given. Each column represents three experiments and the bars are the range. a, Clone A cells (5×10^4); b, T cells (5×10^4); c, B cells (5×10^4); d, B cells (1.5×10^4) plus T cells (3.5×10^4) ($P < 0.05$); e, B cells (1.5×10^4) plus clone A cells (3.5×10^4) ($P < 0.05$). Expt 2: f, clone A cells (5×10^4); g, T cells (5×10^4); h, unfractionated normal spleen cells (5×10^4); i, unfractionated normal spleen cells (5×10^4) plus T cells (5×10^3) ($P < 0.05$); j, unfractionated normal spleen cells (5×10^4) plus clone A cells (5×10^3) ($P < 0.05$); k, unfractionated normal spleen cells (5×10^4) plus clone A cells (1.5×10^4) ($P < 0.05$). Expt 3: l, B cells (5×10^4); m, B cells (5×10^4) plus T cells (5×10^3) ($P < 0.05$); n, B cells (5×10^4) plus T cells (1.5×10^4) ($P < 0.01$); o, B cells (5×10^4) plus clone A cells (5×10^3) ($P < 0.01$); p, B cells (5×10^4) plus clone A cells (1.5×10^4) ($P < 0.01$).

MSV in young BALB/c mice^{10,13}. Unexpectedly, transformed Ly 1⁺ T cells were repeatedly cloned *in vitro* from cell suspensions derived from tumour infiltrates of this new B6 lymphoma and retained at least one T-cell function, the enhancement of immunoglobulin secretion by B cells cultured in the absence of antigen. Inoculation of the T-cell clones into normal B6 mice resulted in rapidly proliferating (and fatal) large cell lymphoma histologically dissimilar to the B-cell lymphoma (STC).

At least two models could explain our data. In the first, the μ -chain positive B cell is primarily transformed by MuLV-M, the retrovirus associated with MSV. This transformed cell, which sheds both virus and immunoglobulin selectively, activates and expands a subpopulation of Ly 1⁺ T cells. These T cells may then become either transformed (with their proliferation suppressed) or pre-malignant. Their full malignant potential may not be realized until direct *in vitro* cultivation, which permits the proliferation or ultimate transformation by MuLV. An alternative model is that the STC contains a small population of malignant Ly 1⁺ T cells which are necessary to maintain (and transfer) the tumour complex *in vivo*. In this case, the IgM⁺ B cells (which are most numerous) are a secondarily recruited and stimulated population. It could be argued that the B-cell enrichment and T-cell depletion procedures are insufficient to remove the T cells. On *in vitro* culture, the malignant T cells outgrow the B cells which on reinjection, give rise to a distinct tumour pattern.

The work reported here and earlier research showing that certain T-cell lymphomas may retain cytotoxic T-cell function¹⁴ demonstrate the necessity of isolating and functionally characterizing lymphocyte subpopulations which exist within a tumour cell population. This approach may be useful investigating the

immunological regulation of the proliferation and differentiation of malignant cells during lymphomagenesis.

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Monoclonal anti-Fc IgG receptor antibodies trigger B lymphocyte function

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Despite intensive investigation, it remains unclear which receptor(s) on the B lymphocyte receives the signal(s) which leads to differentiation and antibody secretion. Binding of ligand (that is, specific antibodies) to the antigen receptors IgM or IgD, in the absence of T lymphocytes or their soluble products, leads to proliferation of normal B cells but not to antibody secretion^{1,2}. Based on the observation that IgG Fc receptors (FcγR) have specific and unique interactions with other B-lymphocyte surface membrane molecules including *I*-region antigens³, IgM⁴ and IgD⁵, it was suggested that FcγR assist in regulation of B-lymphocyte responses⁵. Here we demonstrate that monoclonal antibodies specific for FcγR trigger normal B cells both to proliferate and to secrete antibody in the absence of T lymphocytes. These data suggest that the functional activation of B lymphocytes during normal immune responses may be mediated by FcγR.

2.4G2, a hybridoma that has been extensively characterized^{6–8}, was produced by fusing the mouse myeloma P3U1 with spleen cells from a rat immunized with the mouse macrophage-like cell lines J774 and P388D1. The antibody produced by 2.4G2 is a rat IgG specific for the macrophage FcγR which binds mouse IgG complexes of IgG1 and IgG2b subclasses and is trypsin resistant. In addition, due to antigenic relatedness, 2.4G2 recognizes the FcγR of lymphocytes as assessed using normal spleen cells and lymphoid cell lines. The antigen recognized is non-polymorphic in that it is present in all mouse strains tested. Evidence that 2.4G2 is specific for FcγR can be summarized as follows: (1) 2.4G2 Fab only binds to FcγR-positive cells; (2) it inhibits binding of IgG complexes to FcγR; (3) 2.4G2 Fab binds to a single cell-surface glycoprotein which, when isolated, binds IgG complexes; and (4) the latter binding can again be inhibited by 2.4G2 Fab.

The 2.4G2 hybridoma cell line was grown in tissue culture in standard conditions, and 2.4G2 IgG was isolated from the supernatants by affinity chromatography over goat anti-rat IgG linked to Sepharose. All preparations used in the experiments reported here were pure as judged by SDS-polyacrylamide gel

electrophoresis. The immunoglobulin was typed as a rat $\gamma 2b$ by Ouchterlony analysis with subclass-specific reagents (data not shown).

As a first step towards the evaluation of its functional effects, we examined the binding of 2.4G2 to the Fc γ R of normal

spleen B lymphocytes. Binding of 2.4G2 to normal spleen cells could be readily detected using fluorescein isothiocyanate (FITC)-conjugated, affinity-purified mouse anti-rat IgG and flow microfluorometry; 55–70% of the cells were positive in several experiments compared with 2–3% positive cells in the controls (rat IgG or the detection reagent alone). Double labelling of normal mouse spleen cells for surface IgM and 2.4G2 revealed that 99% of IgM-positive spleen cells (B lymphocytes) bound 2.4G2 (Fig. 1a). Moreover, preincubation with 2.4G2 almost totally (98%) inhibited the binding of IgG complexes to normal spleen cells (Fig. 1b). In the conditions used, most of the cells that bind IgG complexes are B lymphocytes³. Thus, it seems that 2.4G2 binds to the Fc γ R of normal spleen B lymphocytes.

We then cultured T lymphocyte-depleted normal spleen cells with 2.4G2 and measured the proliferative and plaque-forming cell (PFC) responses. T lymphocyte-depleted populations were chosen because T cells can produce factor(s) which assist in B-cell responses^{9–11}. B cells proliferated and generated PFC when cultured with 2.4G2 (Fig. 2). The magnitude of these responses was quite remarkable, being similar to those seen with the mitogen lipopolysaccharide (LPS), although some variability between experiments was noted (see Table 1). Responses were observed at concentrations of 2.4G2 as low as $3 \mu\text{g ml}^{-1}$ (not shown) but maximum responses required 90–100 $\mu\text{g ml}^{-1}$ (Fig. 2). It is not clear why these relatively high concentrations are required although similar observations have been made in other systems (for example, anti- μ stimulation of B-cell proliferation¹). Experiments are being carried out to examine possible explanations of this requirement: for example, receptor turnover, antibody degradation, and mode of presentation.

We then investigated the specificity of the response (Table 1). Neither rat IgG nor two rat monoclonal antibodies which react with surface molecules of B cells evoked any response. One of the monoclonal antibodies, 14D10, is particularly relevant as it has the same isotype as 2.4G2 (ref. 12). Rabbit F(ab')₂ anti-mouse μ , as reported by others^{1,2}, induced proliferation but not plaque-forming cells (PFC), which suggests that the fetal calf serum used did not contain differentiation promoting factors. Other control experiments (not shown) demonstrated

Table 1 Response of T lymphocyte-depleted normal spleen cells to 2.4G2 in specific

Expt	Reagent	Conc ($\mu\text{g ml}^{-1}$)	³ H-TdR incorporated	PFC
a	2.4G2	100	102,334 (1.06)*	65,567 (1.10)*
		30	79,367 (1.04)	43,832 (1.01)
	14D10	100	5,486 (1.07)	1,858 (1.07)
		30	5,089 (1.02)	2,209 (1.04)
	Rat IgG	100	4,628 (1.02)	2,117 (1.07)
		30	5,286 (1.02)	1,742 (1.19)
b	LPS	50	61,030 (1.02)	77,198 (1.10)
		—	5,478 (1.09)	1,567 (1.16)
	2.4G2	90	44,280 (1.07)	12,880 (1.12)
		30	19,669 (1.05)	6,000 (1.02)
	B25-1	30	3,706 (1.03)	850 (1.06)
		90	3,559 (1.07)	1,010 (1.21)
c	Rat IgG	30	4,415 (1.01)	1,380 (1.14)
		50	81,289 (1.06)	52,480 (1.02)
	Medium alone	—	4,738 (1.03)	840 (1.04)
		75	49,919 (1.03)	37,120 (1.11)
	2.4G2	200	43,643 (1.09)	560 (1.18)
		50	61,820 (1.02)	65,300 (1.10)
d	Rabbit F(ab') ₂ anti-mouse μ	—	3,982 (1.14)	850 (1.37)
		—	—	—

Cell preparation, culture and assay conditions were as described in Fig. 2 legend. The strain used was DBA/2J (a and b), or CBA/J (c). Mice were 2–3 months old. 2.4G2 was prepared as described in the text. 14D10 was prepared similarly and is a rat $\gamma 2b$ monoclonal antibody (subclass identical to 2.4G2) which binds to mouse B cells¹². B25-1 is a rat $\gamma 2a$ monoclonal antibody which reacts with H-2D^d and was prepared as described elsewhere⁸. Rabbit F(ab')₂ anti-mouse μ (heavy chain-specific) was prepared as described previously⁴. Rat IgG was from Cappel Laboratories, Pennsylvania). ³H-TdR, ³H-labelled thymidine. PFC, plaque-forming cells.

* Geometric means (c.p.m. per well and PFC per well) of triplicate cultures. Standard errors of the means are shown in parentheses.

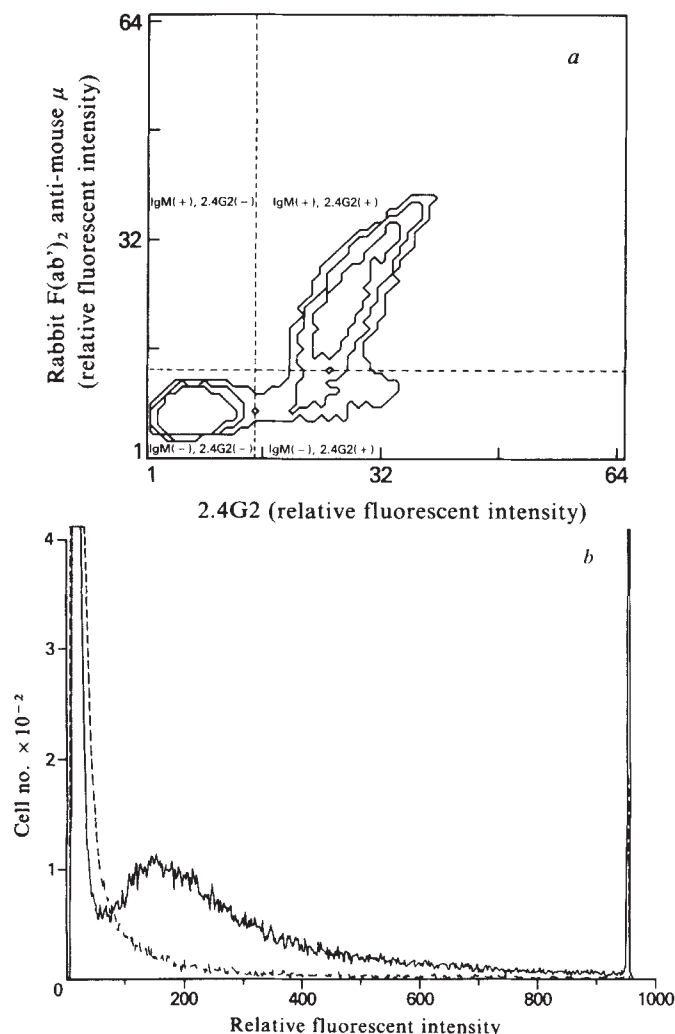


Fig. 1 Flow microfluorometric analysis of 2.4G2 (anti-Fc γ R) binding to normal mouse spleen cells. *a*, Double-label analysis for surface IgM and 2.4G2 demonstrates that 2.4G2 binds to almost all IgM-positive normal spleen cells. A single cell suspension from normal spleen (C57BL/10) was sequentially incubated at 4°C (with thorough washing after each reagent) with saturating amounts of Biotinyl-rabbit F(ab')₂ anti-mouse μ , Texas red avidin, 2.4G2, and FITC-mouse anti-rat immunoglobulin. Flow microfluorometry and data collection were performed as described elsewhere²⁰ using a Becton-Dickinson Dual Laser FACS II, and the two-parameter data are shown as a contour plot. Single-parameter profiles compared with controls were used to establish the cut-off channel for positivity (shown by broken lines). Percentage positive cells: 44.33 for IgM and 56.12 for 2.4G2. Comparison of single marker controls with the individual markers in the double label experiment showed no essential differences, ruling out both cross-reactions between reagents and inhibition of binding of one ligand by the other. Individual channel analysis revealed that of the IgM-positive cells, 99.19% were positive for 2.4G2. Of the 2.4G2-positive cells, 78.35% were positive for IgM. *b*, Inhibition of binding of IgG complexes to normal mouse spleen cells by 2.4G2. A single cell suspension from normal spleen (B10.A) was incubated with a saturating amount of 2.4G2 (broken line) or medium alone (solid line), washed, then incubated with FITC-conjugated heat-aggregated human IgG³. Data are shown as cell number at a given intensity versus relative fluorescent intensity. Inhibition of binding of IgG complexes by 2.4G2 was 97.9% as calculated from the median fluorescence using unlabelled cells as a negative control (not shown).

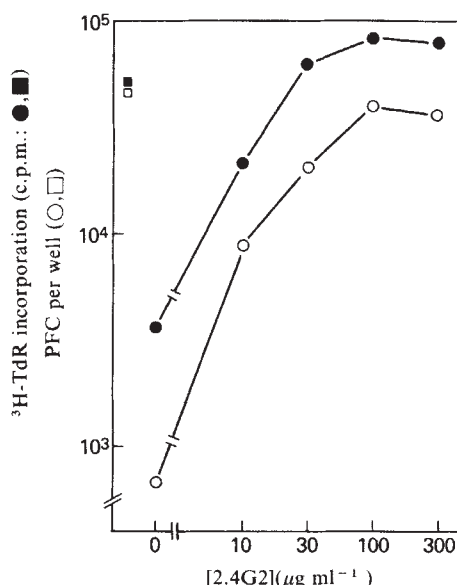


Fig. 2 Proliferative and plaque-forming cell (PFC) responses of T lymphocyte-depleted normal spleen cells to 2.4G2. Single cell suspensions from normal spleen (DBA/2J) were depleted of T lymphocytes by treatment with anti-Thy 1 and anti-Lyt 1 monoclonal antibodies followed by complement (the proliferative responses of such populations to concanavalin A were at background level or below). T lymphocyte-depleted normal spleen cells were cultured (2×10^5 cells in 200 µl per microculture well) in Iscove's modified Dulbecco's medium containing sodium bicarbonate (30 mM), β -mercaptoethanol (5×10^{-5} M), penicillin (100 IU ml^{-1}), streptomycin (100 µg ml^{-1}), and fetal calf serum (10% v/v), with various amounts of 2.4G2 (circles) or 50 µg ml^{-1} LPS (squares) for 3–4 days. Proliferation (solid symbols) was measured by ^3H -TdR incorporation (1 µCi per well for the last 6 h of culture) at day 3. PFC responses (open symbols) were measured at day 4 using Protein A-coupled sheep red blood cells and rabbit anti-mouse immunoglobulin (polyvalent) as developing reagent²¹. Numbers shown are c.p.m. per well and PFC per well. Points are the geometric means of triplicate cultures. Standard errors of the mean were 2–9% (not shown).

that: (1) all plaques contained in their centres lymphocytes, as evaluated microscopically; (2) incubation of fresh T lymphocyte-depleted normal spleen cells with 2.4G2 just before the PFC assay did not induce any PFC, hence excluding any potential interaction between 2.4G2 and the assay reagents; and (3) cells from the CBA/N strain (which lacks a subpopulation of B lymphocytes¹³) responded to LPS but not to 2.4G2. This latter experiment argues strongly against endotoxin contamination of the 2.4G2, and also suggests that the LyB5-positive B cells are those responsive to anti-FcγR antibodies (to be published). It was concluded that the responses to 2.4G2 are specific.

The finding that monoclonal anti-FcγR antibodies evoke B-cell proliferation and antibody secretion raises a number of questions which are now being investigated. (1) Although T lymphocytes were not required, what effect will they have on these responses? (2) What is the role, if any, of macrophages/accessory cells? (3) Is either the Fc portion of 2.4G2 or divalency required? (4) Which subpopulation(s) of B lymphocytes is responding to 2.4G2? (5) Is the population which proliferates the same as that which differentiates to secrete antibody?

Perhaps the most important question raised by these results is: What is the physiological ligand for which 2.4G2 is a model? It seems unlikely that IgG complexes accomplish this, as they exert negative effects on B-cell function in many systems^{14,15}. It also seems unlikely that the macrophage-produced Fc-derived fragment described by Weigle and associates^{16–19} is the ligand. First, Fc-derived fragment induces B-lymphocyte proliferation but has an absolute requirement for T cells in order

to evoke PFC¹⁸, while 2.4G2 does not. Second, Fc-derived fragment-induced B-cell proliferation is absent from the C3H/HeJ strain (due to a macrophage defect)¹⁹, whereas 2.4G2 elicits B-cell function in this strain (to be published). We propose instead that B-lymphocyte FcγR are the receptors for T helper cells and/or their products. Experiments to test this hypothesis are in progress.

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De novo DNA synthesis by a novel mouse DNA polymerase associated with primase activity

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Several studies have shown that short nascent DNAs (Okazaki fragments) of mammalian cells^{1–3} and papovaviruses^{4,5} possess an 8–11-nucleotide RNA sequence covalently linked to their 5' end, suggesting that this RNA sequence acts in priming in DNA synthesis. Although many studies have indicated that DNA polymerase α probably participates in the synthesis of Okazaki fragments^{6–10}, to date, the reconstitution of *de novo* DNA synthesis by coupled reaction of DNA polymerase and classical RNA polymerases of mammalian cells has been unsuccessful. Here we report that a novel mouse DNA polymerase associated with primase activity^{11,12} synthesized DNA of ~600 nucleotides after synthesis of initiator RNA (iRNA) of 8–10 nucleotides in the presence of a specific stimulating factor. The similarity of this DNA synthesis to that *in vivo* suggests the involvement of this novel DNA polymerase in the reaction required to initiate synthesis of Okazaki fragments in chromosomal DNA replication.

We found previously that a subspecies of mouse DNA polymerase α was co-purified with a novel RNA polymerase activity by successive ion-exchange column chromatographies and that it could synthesize DNA with poly(dC) and poly(dT) as template without added primer in the presence of a specific stimulating factor and complementary ribonucleoside triphosphate (rNTP)¹¹. Detailed analysis of the reaction synthesizing DNA and RNA indicated that RNA synthesized by the associated RNA polymerase activity is active in priming DNA synthesis: analysis of the products synthesized on poly(dT) by nearest-

Table 1 Requirement for DNA synthesis by DNA replicase with single-stranded circular fd DNA

Component omitted	dTMP incorporated (pmol)	(%)
None	71	100
fd DNA	0.2	0.3
SF	17.7	24.9
SF, 4NTPs	1.63	2.3
4NTPs	0.34	0.5
ATP	19.8	27.9
GTP	66.6	93.8
CTP	64.0	90.1
UTP	56.4	79.4
ATP, GTP	7.61	10.7
ATP, CTP	6.23	8.8
GTP, CTP	31.3	44.1
CTP, UTP, GTP	23.9	33.7

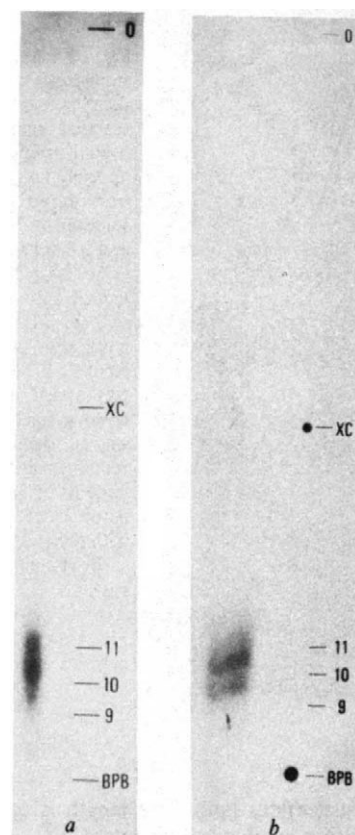
DNA replicase and the stimulating factor (SF) were purified from Ehrlich ascites tumour cells as described earlier¹¹. Phage fd single-stranded DNA was extracted from the phage with phenol saturated with 50 mM sodium tetraborate (pH 9.0). The complete reaction mixture (50 μ l) contained 25 mM Tris-HCl (pH 7.6), 40 mM KCl, 1 mM dithiothreitol, 2 μ g of bovine serum albumin, 2 mM MgCl₂, 12.7 μ M ³H-dTTP (140 c.p.m. pmol⁻¹), 125 μ M each of dATP, dGTP and dCTP, 300 μ M each of ATP, CTP, GTP and UTP, 25 μ g ml⁻¹ fd DNA, 24 ng of the stimulating factor and 0.4 units¹¹ of DNA replicase. After incubation at 37 °C for 30 min, 40 μ l of the mixture was spotted onto a glass filter (Whatman GF/C) and processed as described previously to measure the radioactivity incorporated into trichloroacetic acid-insoluble material²².

neighbour transfer of ³²P from α -labelled nucleoside triphosphates showed that the short RNA (8–10 nucleotides long) is covalently linked to the 5' end of the DNA product molecule and furthermore that the associated RNA polymerase activity can incorporate deoxynucleotides into iRNA, although to only a limited extent¹³. Thus, this novel RNA polymerase activity resembles *Escherichia coli* primase¹⁴ in some features.

Although it is unknown whether primase activity is catalysed by the same protein as DNA polymerase activity, for convenience, we named this DNA polymerase associated with primase activity 'DNA replicase', to distinguish it from the major species of DNA polymerase α that is unable to use unprimed single-stranded template. Together with previous findings that the DNA replicase activity is localized in the nuclear fraction and has a positive relationship with DNA replicative activity of cells in tissues¹⁵, the novel features of the DNA replicase activity suggest that it participates in the discontinuous synthesis of chromosomal DNA.

To obtain more insight into the role of DNA replicase in discontinuous chromosomal DNA replication, we used physiological DNA, single-stranded circular fd DNA, as template instead of synthetic homopolymer. Table 1 shows that DNA synthesis on fd DNA was largely dependent on the addition of both the stimulating factor and four ribonucleoside triphosphates, but that in the presence of ATP alone, substantial DNA synthesis was still maintained. Next, to determine the size of iRNA, the products labelled with [α -³²P]ATP (Fig. 1a) or [γ -³²P]ATP (Fig. 1b) were digested with DNase I and subjected to electrophoresis on a polyacrylamide gel in 7 M urea. The RNA released by this treatment migrated in the p(Ap)_{10–11} marker range. However, corrections should be made in calculating the size of iRNA from the mobility, because the RNAs possessed a triphosphate group at their 5' end (Fig. 1b) and one to three deoxynucleotides at their 3' end⁴, and marker oligonucleotides possessed 5'-terminal labelled phosphate added by T4 polynucleotide kinase. Assuming that the mobility of oligonucleotide without terminal phosphate is the same as that of oligonucleotide without terminal phosphate but with one nucleotide less¹⁶, the iRNA was estimated to be 8–10

Fig. 1 Size analysis of iRNA synthesized by DNA replicase with fd single-stranded DNA. Polyacrylamide gel electrophoresis in 7 M urea of iRNA released by DNase I digestion of the products is shown. The reaction with DNA replicase was performed in the complete reaction conditions described in Table 1 legend except that 300 μ M of [α -³²P]ATP (a) or [γ -³²P]ATP (b) (1,100 c.p.m. pmol⁻¹) was added as labelled substrate and the mixture was scaled up to 400 μ l. After incubation at 37 °C for 30 min, the reaction was stopped by adding EDTA to 10 mM and the sample was deproteinized with phenol. The products were then separated on a 5–20% sucrose gradient in 5 mM KPO₄, 0.2 mM EDTA; those that sedimented slightly faster than marker fd single-stranded DNA were precipitated by adding ethanol and dissolved in 100 μ l of H₂O. The sample solution was adjusted to 50 mM Tris-HCl (pH 7.6), 20 mM MgCl₂, 15 mM CaCl₂, 100 μ g ml⁻¹ calf thymus DNA, 250 μ g ml⁻¹ yeast RNA and 0.1 mg ml⁻¹ DNase I (Worthington), incubated for 1 h at 37 °C and then heated at 80 °C for 5 min. The digest was dried and the residue was dissolved in 20 μ l of a solution of 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 8 M urea, 0.05% bromophenol blue (BPB) and 0.05% xylene cyanol (XC) and heated at 90 °C for 30 s. The size of iRNA was determined by electrophoresis of the digests on 20% polyacrylamide gel in 7 M urea²³. Electrophoresis was carried out at 300 V for 13 h. The wet gel was then autoradiographed with a Dupont lightning-plus intensifying screen at -80 °C. Chain length markers were prepared by partial alkaline hydrolysis²⁴ of poly(rA) followed by 5'-end labelling with T4 polynucleotide kinase and [γ -³²P]ATP²⁵.

**Table 2** Nearest-neighbour analysis with ³²P transfer from [α -³²P]dATP to 2'(3')NMP

Ribonucleotide	c.p.m.	%
Ap	15,566	32.0
Gp	13,056	26.9
Up	13,095	27.0
Cp	6,840	14.1
Total	48,557	100

The products were synthesized in the complete reaction mixture described in Table 1 legend except that the concentrations of dNTP were reduced to 25 μ M and 150 μ M, respectively, and [α -³²P]dATP was added instead ³H-dTTP. The products isolated as described in Fig. 1 legend were hydrolysed with 0.4 M NaOH at 37 °C for 16 h and then neutralized with Dowex 50W (H⁺ form). After addition of marker 2'(3') nucleoside monophosphates (NMPs), the digest was adjusted to 7 M urea, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA and applied to a DEAE-Sephadex A-25 column (0.3 × 70 cm) pre-equilibrated with the above buffer. Nucleotides were eluted from the column with a linear gradient of 0–0.5 M NaCl in the above buffer (500 ml). The fractions containing mononucleotide were collected, diluted with distilled water and then applied to a Dowex AG-1 column (0.3 × 15 cm). After washing with 0.004 M HCl, nucleotides were eluted with a linear gradient of 0.004–0.01 M HCl/0.1 M NaCl (40 ml). ³²P radioactivity was measured in ACS-II scintillator (Amersham).

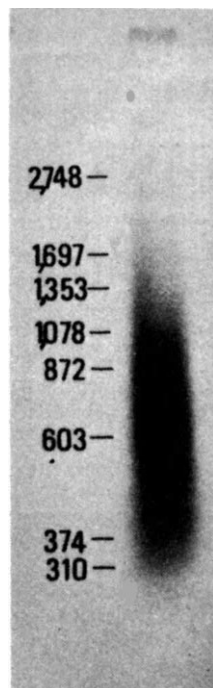


Fig. 2 Alkaline agarose gel electrophoresis of the DNA products. The reaction was performed in the complete reaction conditions described in Table 1 legend except that the mixture was scaled up to 400 μ l. The sample was deproteinized, mixed with an equal volume of a solution of 30% glycerol, 0.1 M NaOH and 0.3% bromocresol green and heated at 60 °C for 5 min. The sample was then loaded onto an alkaline agarose vertical slab gel (11 $\times$ 18 $\times$ 0.3 cm) prepared from a solution of 1.4% agarose in 30 mM NaOH, 2 mM EDTA. Electrophoresis was carried out at 8 mA until the bromocresol green had migrated $\sim$ 8 cm. The gel was autoradiographed by the method of radiofluorography described by Chamberlain²⁶. Chain length markers were prepared from ϕ X174 DNA by digestion with either *Hae*III or *Hpa*II and detected by staining with ethidium bromide.

nucleotides long. This length is compatible with the size of iRNA synthesized on poly(dT)¹³.

We next examined the length of the DNA products by alkaline agarose gel electrophoresis (Fig. 2). The mean length of DNA was $\sim$ 600 nucleotides, longer than reported for short nascent DNAs synthesized in intact cells^{1,2} and DNA fragments synthesized by mammalian DNA polymerase α on restriction fragment-primed ϕ X174 single-stranded DNA^{17,18}. The length of DNA fragments synthesized by *Drosophila* DNA polymerase α on primed M13 DNA approximates to this length when single-stranded DNA binding protein is added¹⁹. Northern hybridization analysis showed a nearly equal distribution of the location of DNA synthesis of fd DNA.

In polyoma virus DNA replication *in vitro*, the ribonucleotide residue at the iRNA-DNA junction of short nascent DNA was found to be random²⁰. To determine the ribonucleotide distribution at iRNA-DNA junctions, the products synthesized with [α -³²P]dATP by DNA replicase were hydrolysed with alkali, and the monoribonucleotides released by this treatment were analysed by column chromatography on DEAE-Sephadex in 7 M urea and on Dowex 1. Table 2 shows that all four ribonucleotides were present at the junctions adjacent to dAMP, although the frequency of AMP was somewhat higher and that of CMP lower than those of GMP and UMP. These results suggest that iRNA synthesis was either initiated or terminated at random sites on fd DNA. Only small amounts of oligonucleotides larger than dinucleotide other than 5'-terminal ribonucleoside triphosphates were detected on DEAE-Sephadex chromatography of an alkaline hydrolysate of the products synthesized at the rNTP to dNTP ratio described in Table 2 legend. This indicates that the primase activity incorporated only small amounts of deoxynucleotides into iRNA at low dNTP concentrations, as it did in the reaction with poly(dT)¹³.

The size of iRNA synthesized by the associated primase activity is compatible with values observed by others in chromosomal DNA replication of intact cells³ and isolated nuclei⁵ and in papovavirus DNA replication in isolated nuclei of infected cells^{4,5}. Our finding of this novel DNA synthesis on unprimed fd DNA by mouse DNA replicase is the first step in the establishment of a reconstitution system for DNA replication and thus for the study of the mechanism of discontinuous replication of chromosomal DNA in mammalian cells.

Novel RNA polymerase activity that synthesizes iRNA of unique size was recently detected by Kaufmann and Falk²¹ in extracts from SV40-infected cells.

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Phosphorylation of troponin I and phospholamban during catecholamine stimulation of rabbit heart

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During adrenergic stimulation heart cells develop increased force and there is an increase in the rate of rise and fall of force. These changes are thought to be triggered by the binding of catecholamine to the β -receptor, which in turn signals alterations in membranes and the contracto-regulatory protein complex by multiple, coordinated mechanisms involving Ca^{2+} , cyclic nucleotides and protein phosphorylation^{1,2}. This view is supported by results of *in vitro* studies with cardiac myofibrils^{3,4} and vesicles of sarcoplasmic reticulum (SR)⁵⁻⁷ showing that these organelles have sites of protein phosphorylation that may act as effectors of the adrenergic signal giving rise to the altered twitch dynamics. Phosphorylation of troponin I (TnI), a regulatory protein of cardiac myofibrils, results in a decreased steady-state affinity of troponin (TnC) for Ca^{2+} , an increase in the off rate for Ca^{2+} exchange with TnC⁸ and a rightward shift of the relationship between free Ca^{2+} and myofibrillar force⁹ or ATPase^{3,4}. Phosphorylation of phospholamban, a regulatory protein of cardiac SR, increases the velocity of Ca^{2+} transport by SR vesicles, the affinity of the transport protein for Ca^{2+}

and the turnover of elementary steps of the ATPase reaction^{5-7,10,11}. Taken together, these findings support the hypothesis that the inotropic response of the heart to catecholamine stimulation involves phosphorylation of TnI and phospholamban. Yet, although it is known that TnI is phosphorylated during adrenergic stimulation of beating heart¹²⁻¹⁴ there has been no clear evidence that phospholamban is phosphorylated at the same time. We now report that during the peak of the inotropic response of the rabbit heart to perfusion with isoproterenol, there is simultaneous phosphorylation of TnI and an 11,000-molecular weight (M_r) protein associated with SR, which is probably monomeric phospholamban.

Hearts removed rapidly from stunned rabbits were perfused retrogradely with modified Krebs-Henseleit buffer in a Langendorff-type apparatus and we recorded the force and heart rate as described previously^{13,15}. We perfused the hearts for 5 min without recirculation, and then for 15 min by recirculation with buffer containing $10 \mu\text{Ci ml}^{-1}$ ^{32}P -labelled orthophosphate and $10 \mu\text{M}$ 1-methyl-3-isobutylxanthine (MIX). After the perfusion with ^{32}P , control hearts were perfused for 60 s with $1 \mu\text{M}$ isoproterenol, at which time the force of contraction was maximally stimulated. The hearts were freeze-clamped with Wollenberger clamps at the temperature of liquid nitrogen. The frozen hearts were powdered under liquid nitrogen, and myofibrils and SR vesicles were isolated from the same frozen heart powder. Microsomes enriched in SR vesicles were prepared as previously described¹⁶ with the modifications described in Table 1 legend. The isolation buffer contained $30 \text{ mM KH}_2\text{PO}_4$ (pH 7.0), 70 mM NaF and 5 mM EDTA to inhibit phosphorylation and dephosphorylation reactions during the extraction. Functional cardiac myofibrils retaining covalent phosphate were purified as previously described¹⁷, using the pellet obtained after the first centrifugation of heart homogenate as starting material.

SR vesicles supported Ca^{2+} uptake in the presence of 5 mM oxalate ($0.15 \mu\text{mol Ca}^{2+} \text{ mg min}^{-1}$). The ATPase activity

was $0.35\text{--}0.5 \mu\text{mol P}_i \text{ mg min}^{-1}$ and was not sensitive to ouabain ^3H -ouabain binding assays¹⁸ revealed up to $1.5 \text{ pmoles } ^3\text{H}$ -ouabain bound per mg SR compared with $300 \text{ pmoles } ^3\text{H}$ -ouabain bound per mg of purified sarcolemma (SL)¹⁹ in the same experimental conditions. Sarcolemma contamination was further estimated by dihydropyrenol binding ($140 \text{ fmol per mg SR}$ compared with $1,346 \text{ fmol per mg SL}$), quinuclidinyl benzilate binding ($289 \text{ fmol per mg SR}$ compared with $1,850 \text{ fmol per mg SL}$) and adenylate cyclase activity assayed in the presence of 10 mM NaF ($401 \text{ pmol per min per mg SR}$ compared with $4,088 \text{ pmol per min per mg SL}$). The activities of the enzyme markers in SR were $\sim 10\%$ of the levels present in rabbit cardiac SL purified as previously described¹⁹. Mitochondrial contamination was estimated from the levels of cytochromes $a + a_3$ present in SR and from the NaN_3 sensitivity of the ATPase activity to be $\sim 35\%$. However, the mitochondrial content of the microsomes prepared from different hearts was nearly constant.

Table 1 summarizes the amount of acid-precipitable ^{32}P , incorporated into SR vesicles isolated from six pairs of control and isoproterenol-stimulated hearts. In all cases, there was an increase in the acid-stable ^{32}P counts in the precipitates obtained from the stimulated hearts. This is due to a net increase in SR phosphorylation as we could find no evidence for differences in ^{32}P specific activity in paired control and stimulated hearts. Normalization of the data with respect to control hearts shows that perfusion of the hearts with isoproterenol resulted in a 1.98 ± 0.26 -fold increase in ^{32}P -phosphate incorporation.

We further analysed the SR vesicles prepared from control and stimulated hearts by SDS-polyacrylamide gel electrophoresis and autoradiography. The results shown in Fig. 1 are typical of six experiments with paired control and stimulated hearts and indicate that (1) the pattern and relative proportions of microsomal proteins were the same in each preparation; (2) essentially all the ^{32}P -phosphate incorporated into the SR vesicles was associated with an 11,000- M_r protein; and (3) incorporation of ^{32}P into this protein increased on stimulation of the hearts with isoproterenol. In view of *in vitro* results using cardiac SR vesicles²⁰⁻²², the 11,000- M_r protein is probably the monomeric form of phospholamban. These results are consistent with previous reports of the presence of an 11,000- M_r phosphoprotein in microsomes isolated from ^{32}P -perfused guinea pig²³ and rat²⁴ hearts.

Table 1 Phosphorylation of rabbit cardiac sarcoplasmic reticulum in control and isoproterenol-stimulated beating hearts

Expt	^{32}P incorporation (c.p.m. per mg SR)	
	Control	Stimulated
1	2,025	4,540
2	2,721	3,980
3	641	1,770
4	2,081	2,812
5	760	1,080
6	2,518	3,831

SR vesicles were isolated from control and isoproterenol-stimulated rabbit hearts. The frozen powdered tissue was simultaneously thawed and homogenized in medium A ($30 \text{ mM KH}_2\text{PO}_4$, (pH 7.0), 70 mM NaF , 5 mM EDTA , 0.3 M sucrose) containing 0.3 mM phenylmethyl sulphonyl fluoride, 0.5 mM MIX and 0.5 mM dithiothreitol. The homogenate was centrifuged at $4,300g$ (R_{max}) for 10 min. The resultant supernatant fraction was passed through four layers of cheesecloth and the filtrate was centrifuged at $15,000g$ (R_{max}) for 20 min. The supernatant fraction was again passed through four layers of cheesecloth and centrifuged at $143,000g$ (R_{max}) for 30 min. The pellet was suspended in medium A containing 500 mM KCl and centrifuged at $143,000g$ (R_{max}) for 45 min. The resulting pellet was washed in medium A and the final pellet was suspended in medium A. The protein was adjusted to the same concentration and aliquots (0.05 ml) of each preparation were used to determine the acid-precipitable ^{32}P , incorporated¹⁰. The values shown are the arithmetic means of three determinations with each experimental preparation. Stimulated hearts show a significantly ($P = 0.01$) higher ^{32}P content than control hearts (paired *t*-test).

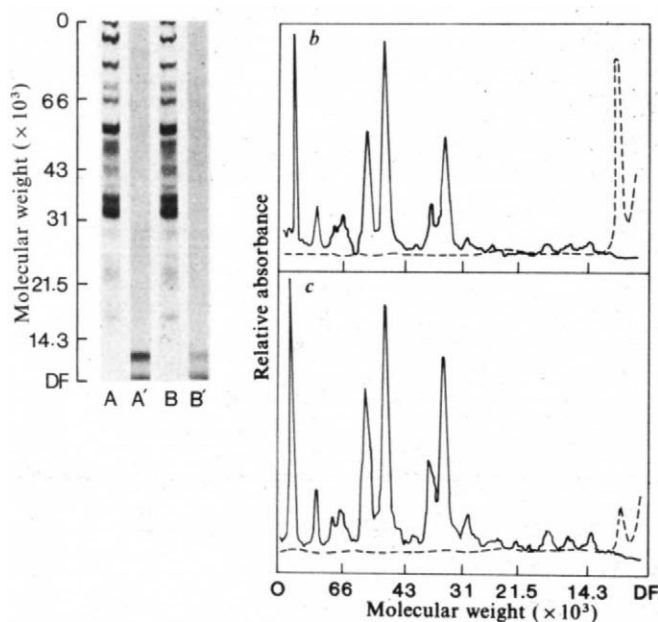


Fig. 1 SDS (0.1%)-polyacrylamide (12%) gel electrophoresis and autoradiography of rabbit cardiac SR ($60 \mu\text{g}$) isolated from rabbit hearts perfused with ^{32}P . *a*, Photographs of Coomassie blue-stained gel and autoradiogram of SR isolated from isoproterenol-stimulated (A, A') and control (B, B') hearts. *b*, *c*, Densitometric scans at 540 nm of gels (—) and autoradiogram (---) of SR preparation from isoproterenol-stimulated (*b*) and control (*c*) rabbit hearts. O, Origin; DF, dye front. Molecular weight markers were human serum albumin ($66,000$), ovalbumin ($43,000$), DNase I ($31,000$), soybean trypsin inhibitor ($21,500$) and egg white lysozyme ($14,300$).

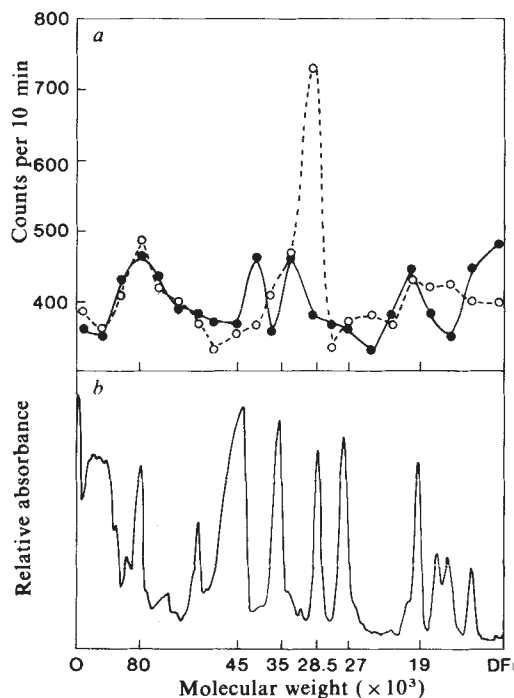


Fig. 2 SDS (0.1%)-polyacrylamide (12%) gel electrophoresis and ^{32}P incorporation of myofibrillar proteins prepared from rabbit hearts. *a*, ^{32}P incorporation into myofibrils from control (—) and isoproterenol-stimulated (---) hearts. *b*, Densitometric scan of myofibrillar preparations stained with Coomassie blue. ^{32}P incorporation was measured in gel slices and the electrophoretic protein profile was the same in control and stimulated hearts. Major myofibrillar proteins (M_r) are actin (45,000), tropomyosin (35,000), troponin I (28,500), light chain I (27,000) and P-light chain (19,000).

We also analysed myofibrils prepared from the same hearts used to study SR phosphorylation. Figure 2 shows densitometric profiles and ^{32}P incorporation into myofibrillar proteins. ^{32}P was incorporated into several of the myofibrillar proteins, but the largest and most consistent increase in ^{32}P incorporation with catecholamine stimulation was into TnI (M_r 27,500). This agrees with results of earlier experiments using TnI extracted from cardiac homogenates by affinity chromatography^{13–15}. There was also ^{32}P incorporation into the P-light chain of myosin in myofibrils prepared from both control and catecholamine-stimulated hearts. However we found no change in the ^{32}P incorporation into the P-light chain with inotropic state. This is consistent with our previous results and those of High and Stull²⁵ and Westwood and Perry²⁶ but contrasts with the findings of Kopp and Bárány²⁷, who reported an increase in P-light chain phosphorylation with positive inotropy. There was no detectable incorporation of ^{32}P into the 27,000- M_r myosin light chain.

The results presented here and elsewhere^{1,2} support the hypothesis that the relaxant effect of catecholamines on heart is due in part to protein phosphorylations of regulatory components of myofibrils and SR. This hypothesis originated from *in vitro* evidence that cyclic AMP-protein kinase-mediated phosphorylation of TnI^{8,9,17} and phospholamban^{5–7} altered the functional properties of myofibrils and SR in a way suggestive of a role in the relaxant effect. A correlation between phosphorylation of TnI and the onset of the positive inotropic effect of catecholamines in beating hearts has been established^{12–15}. Now we report that an 11,000- M_r SR protein is also phosphorylated at the peak of the positive inotropy in beating rabbit hearts. Our enzyme marker measurements indicate minimal contamination of our SR preparations with sarcolemmal membranes and thus argue strongly that the 11,000- M_r phosphoprotein in our preparations originates from the SR. Yet, as with all studies of this kind, we cannot definitely establish that the 11,000- M_r protein existed in the SR in the beating heart.

A further correlation supporting the above hypothesis is that the mechanical response of hearts to catecholamines is unique among muscle types and the sites participating in the phosphorylations described here are unique to heart muscle. Phospholamban is a protein that seems to be present only in cardiac SR preparations² and cardiac TnI is specialized in that it contains an extra 26 amino acids at its N-terminus that are not present in TnI from other types of muscle²⁸. This region of the molecule is phosphorylated *in vitro* on incubation with cyclic AMP and cyclic AMP-protein kinase and *in vivo* in rabbit hearts perfused with adrenaline^{13,15}.

We cannot yet rule out the possibility that although the phosphorylations of TnI and phospholamban are coincidental with the positive inotropic effect of catecholamines, they may not be causal in the altered twitch dynamics. However the *in vitro* effects of protein phosphorylation on the functional properties of SR and myofibrillar proteins and the structural specializations of these organelles argue against this possibility.

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Sequences of immunoglobulin $\lambda 1$ genes in a $\lambda 1$ defective mouse strain

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The $\lambda 1$ light chain accounts for about 80% of λ immunoglobulin chains in BALB/c mice, the remainder being $\lambda 2$ and $\lambda 3$ (refs 1, 2). Several inbred strains of mice, most notably the SJL strain, have greatly reduced $\lambda 1$ expression compared with other strains. Genetic analysis of SJL mice indicates there is a *cis*-acting defect linked to the $\lambda 1$ structural gene³. Although this defect reduces both the number of spleen cells expressing

surface $\lambda 1$ and the serum level of $\lambda 1$, the $\lambda 1$ antibodies that are produced appear normal³. A possible explanation for such a defect would be an alteration of the recognition sequences involved in the rearrangement of the variable (V) and constant (C) region genes for $\lambda 1$. However, the defect could reside in other structural elements involving transcription, RNA processing, protein chain folding or light and heavy chain association. Furthermore, a more general regulatory defect must be considered, as SJL mice also appear somewhat depressed in the synthesis of $\lambda 2$ chains^{1,3,4}. As a first approach to the analysis of the genetic defect in SJL mice, we have now analysed the structural genes involved in the formation of $\lambda 1$ chains and have found one change leading to an amino acid replacement in the exon of C $\lambda 1$. Whether this alteration is of functional significance remains to be elucidated; however, analysis of the DNA of several mouse strains confirms on the molecular level that the SJL defect is closely linked to the λ locus.

We have isolated Charon 4A clones containing all the λ genes of SJL mice. The C λ 3-C λ 1- and V λ 1- containing clones were studied in detail using restriction enzyme mapping, electron microscopy and DNA sequencing, to determine if any structural elements of λ 1 genes which might affect λ 1 expression differ between BALB/c and SJL mice. When clones containing the C λ 3-C λ 1 gene cluster and the V λ 1 gene and their flanking sequences, were mapped using the restriction enzymes *EcoRI*, *Bam*HI, *Sac*I, *Hind*III and *Xba*I, no differences were found compared with their BALB/c counterparts.⁵⁻⁷

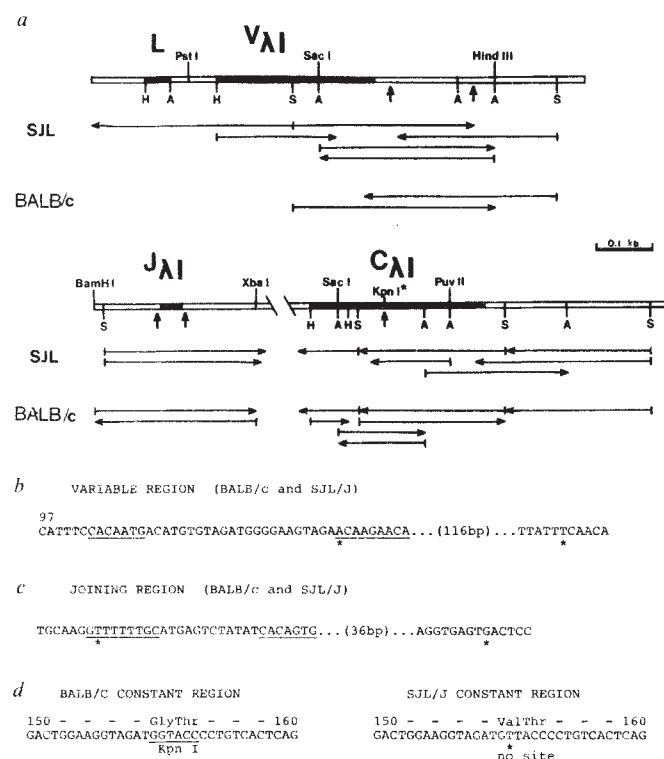


Fig. 1 Comparison of the DNA sequences of SJL and BALB/c λ 1 genes. The BALB/c genes were isolated from a cloned library of kidney DNA⁵⁻⁷ and the SJL genes were obtained from a partial *EcoRI* library of kidney DNA cloned into Charon 4A. *a*, Restriction maps and sequencing strategy. Restriction fragments produced with H = *HaeIII*, A = *AluI* and S = *Sau3A* I were cloned into M13mp7 (ref. 17) for DNA sequencing^{6,7}. The *KpnI** restriction site is present in BALB/c but absent from SJL λ 1 DNA. L, leader sequence; V λ 1 variable region gene; J λ 1, joining gene segment; C λ a1 constant region gene. Sequencing strategy for BALB/c J λ 1 is from ref. 6, that for BALB/c C λ 1 is from ref. 7. $\uparrow$ Indicates where the sequences are located which are shown in *b-d*. *b-d*, Only those portions of the DNA sequence are shown here; differences with the published BALB/c sequence⁸ were found. The consensus recognition sequences^{18,19} are underlined, as is the *KpnI* site. Asterisks indicate differences from the published BALB/c sequence.

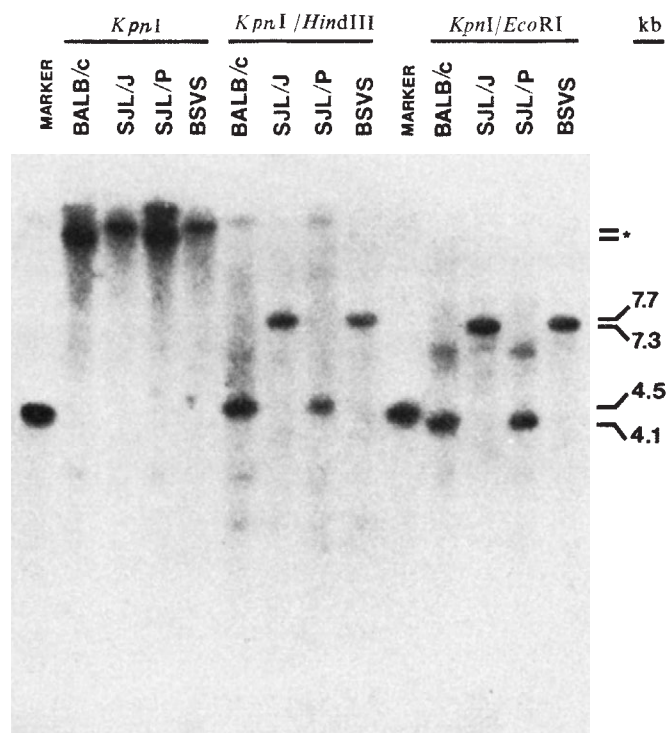


Fig. 2 Southern blot of CA1 genes in genomic DNA of various mouse strains. Only the analysis using *KpnI* is shown. No differences were found using other restriction enzymes. The blot was hybridized with CA1 probe.⁵ In the digests with *KpnI* alone, the size of the large bands cannot be accurately determined, yet the 3.2-kilobase (kb) difference caused by the loss of the *KpnI* site is clearly demonstrated. Some incomplete digestion is evident in the BALB/c and SJL/J DNAs.

(data not shown). Heteroduplex analysis of SJL and BALB/c V λ 1 or C λ 3-C λ 1 genes gave no evidence for sequence heterology, deletions or insertions (data not shown). Heteroduplex analysis in our conditions would not show heterologies smaller than 100 nucleotides in length or less than 20% divergence in sequence⁷.

We determined the DNA sequence of all the exons and the flanking regions which are involved in the formation of functional $\lambda 1$ chains (Fig. 1). From 100 nucleotides upstream of the V λ 1 signal peptide sequence to the 3' end of the V λ 1 exon no differences were found compared with the published BALB/c sequence⁸. Two changes were found in the SJL V λ 1 3'-flanking region, one in the recognition nonamer and one 164 nucleotides downstream from the 3' end of V λ 1. However, when we then sequenced the BALB/c V λ 1 gene to confirm these alterations, the two positions were found to be the same as in SJL (Fig. 1). It is therefore likely that the previously published BALB/c sequence is incorrect. No reason for defective $\lambda 1$ synthesis is apparent from the nucleotide sequence of the SJL V λ 1 gene region.

A total of 300 nucleotides were sequenced in and around the SJL JA 1 gene segment. This region was a prime suspect for a lesion because of its dual role in DNA rearrangement and RNA splicing. Compared with the published BALB/c sequence⁶, differences were found both in the recognition nonamer 5' of and the RNA splice site 3' of the JA 1 exon of SJL (Fig. 1c). However, these two positions were found to be the same when we sequenced the corresponding regions in BALB/c DNA⁶ (Fig. 1). Thus, the JA 1 region itself is apparently not the cause of the defect in SJL.

Finally, the CA1 genes of SJL and BALB/c as well as 27 and 306 nucleotides of the respective flanking sequences 5' and 3' were found to be identical, with one exception. A change from a G in BALB/c to a T in SJL alters the protein sequence of amino acid position 155 from glycine to valine (Fig. 1). The

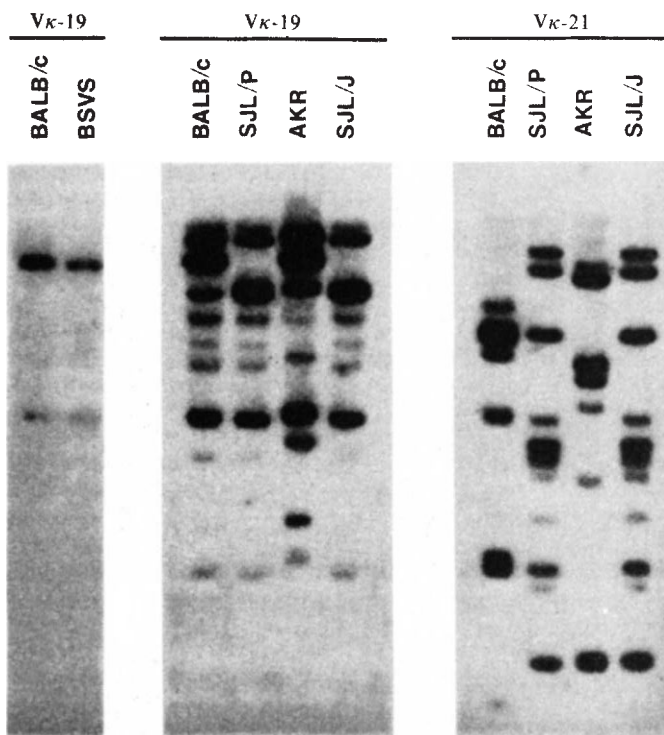


Fig. 3 Southern blots of $V\kappa$ genes in genomic DNA of various mouse strains. The $V\kappa$ -9 probe was a gift of Robert Perry (cDNA clone of κ from myeloma MPC11). The $V\kappa$ -21 probe was a subfragment (Vg) from a genomic clone of the nonfunctional gene of MOPC21²⁰. All DNAs were digested with *Bam*HI. The left-hand panel shows only the most strongly hybridizing bands because the blot was washed under higher than normal stringency.

amino acid change could be the molecular basis for the observed 'allotypic' difference between SJL and BALB/c λ 1 chains^{9,10}. This nucleotide change also eliminates the target site for the restriction enzyme *Kpn*I. Southern blot analysis of genomic DNA (Fig. 2) confirms the presence of the *Kpn*I site in BALB/c DNA and its absence from SJL DNA. This change is therefore not a sequencing error or cloning artefact and is the only difference that we have found between the λ 1 genes of BALB/c and SJL mice.

An alteration of recognition sites presumed to be involved in V-C joining is not borne out by the DNA sequence of the SJL λ 1 genes. It is also likely that, as in $V\kappa$ (ref 11), a promoter for transcription is located within the 100 base pairs (bp) upstream of $V\lambda$ 1, which were found to be normal in SJL. In addition, RNA splice sites are identical to those of BALB/c mice. The *cis*-linked nature of the SJL defect level suggested such possibilities^{3,4}. With the exception of a single base pair change, the entire V and C region exons are identical to the same regions of the BALB/c strain. Although there were no changes in flanking regions we examined, small changes in more distant flanking regions of the λ 1 genes which were not sequenced cannot be ruled out.

Could the low λ 1 synthesis in SJL mice be due entirely to the alteration in λ 1? We have found a striking correlation between the nucleotide change in λ 1 and the levels of λ 1 produced in different mouse strains. The single base pair change at amino acid position 155 in SJL results in a loss of a *Kpn*I site that exists in the BALB/c λ 1 exon. The presence of a *Kpn*I site in λ 1 can be taken as evidence for the BALB/c-like sequence around the amino acid 155 coding position. As shown in Fig. 2, both SJL/J (SJL/Jackson) and BSVS (another strain showing low λ 1 synthesis)³ lack the *Kpn*I site. In contrast, SJL-Potter, a variant SJL strain which produces normal levels of λ 1 (M. Potter and R. Lieberman, personal communication), has a *Kpn*I site at this position. We have confirmed that the SJL-Potter strain is closely related to the SJL-Jackson strain

by restriction mapping of certain $V\kappa$ genes¹². The families of $V\kappa$ -19 and $V\kappa$ -21 genes show strain-specific map differences (Fig. 3). SJL-Jackson and SJL-Potter are identical in their $V\kappa$ -19 and $V\kappa$ -21 patterns and different from both the BALB/c and AKR patterns. These two SJL substrains were separately derived from the original SJL (Swiss Jackson Laboratories) breeding colony of Murphy (M. Potter, personal communication). At that time, the Murphy SJL mice had been inbred for only about 20 generations and presumably contained two different λ 1 genes. The BSVS strain, which produces a low level of λ 1, does not show the $V\kappa$ patterns of SJL/J (Fig. 3). The exact relationship between BSVS and SJL cannot be defined. SJL mice are to a large extent derived from 'Swiss Webster' mice^{13,14}, whereas the BSVS line is derived from bacterium-susceptible and virus-susceptible 'Swiss' mice inbred by Webster¹⁵. Given the close origins of SJL and BSVS strains, the original gene pool from which they were derived could have been heterozygous for $V\kappa$ genes. Subsequently, λ and κ genes may have segregated differently in the two strains. It is likely that the two present day λ 1 genes existed in the original Swiss mouse population.

It is conceivable that the substitution of valine for glycine in λ 1 genes alters the ability of λ chains to associate with certain H chains. The complete three-dimensional structure of the mouse λ 1 protein has not been determined. In a human Kern Oz λ chain, position 155 is present on a loop between segments forming the β -pleated sheets and is not a contact residue with the H chain¹⁶. The substitution of glycine by valine may alter the configuration of the molecule because valine is more hydrophobic and less flexible than glycine. This amino acid change may therefore cause an altered ability of λ chains to bind certain H chains such as γ chains and would fit certain observations in SJL mice. For example, early in immunization, almost normal numbers of λ 1- μ antibody-producing cells are present in SJL mice⁴. At a time after immunization when in normal mice the switch to γ production is almost complete, hardly any λ 1 antibody-producing cells remain in SJL mice⁴. This could mean that, as in normal mice, a switch to γ heavy chains has occurred in SJL λ 1-producing cells, but that the γ chain cannot associate with the λ 1 chain. On the other hand, the fact that in adult SJL mice the levels of λ 1- μ surface immunoglobulin-positive cells are 100 times lower than in BALB/c⁴ is inconsistent with this explanation, unless one invokes additional levels of regulation such as a positive feedback of λ 1- γ production on λ 1- μ surface immunoglobulin-expressing cells. This inconsistency, coupled with the already mentioned reduction in λ 2, may point to a defect in regulation which remains to be explored. Thus, although genetically linked with the λ 1 functional defect, the alteration in λ 1 could simply be a silent polymorphism. Genetic studies are underway in collaboration with M. Potter to determine whether these two traits can be separated.

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Similar genes for a mitochondrial ATPase subunit in the nuclear and mitochondrial genomes of *Neurospora crassa*

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The proton-translocating ATPases from mitochondria, chloroplasts and bacteria consist of ~10 different polypeptide subunits¹. The smallest subunit, a proteolipid of molecular weight ~8,000, is present in a stoichiometric amount of six—these six polypeptides are thought to form a proton channel through the membrane². The proteolipid is affected by the ATPase inhibitors oligomycin and dicyclohexylcarbodiimide (DCCD). As DCCD is covalently bound to it, the proteolipid is commonly referred to as the DCCD-binding protein. Because of the spatial arrangement of the proteolipids in the membrane, binding is probably restricted to one specific glutamic acid residue^{2,3}. In the yeast *Saccharomyces cerevisiae* the DCCD-binding protein is encoded by the mitochondrial DNA and synthesized inside the mitochondria⁴, whereas in *Neurospora crassa*, another ascomycete, the gene for the DCCD-binding protein lies in the nucleus, and the protein is synthesized outside the mitochondria⁵. Here we report the presence on the mitochondrial DNA of *N. crassa* of a nucleotide sequence which potentially encodes another DCCD-binding protein. Although a translation product has not yet been found, we suggest a possible function of this gene and speculate on its evolutionary origin.

We have shown previously by heterologous hybridization that *Neurospora* mitochondrial DNA contains a sequence

homologous to the yeast mitochondrial gene for the DCCD-binding protein⁶. We recently completed the nucleotide sequencing of this fragment (Fig. 1). It contains one open reading frame per strand. The longest reading frame, 225 nucleotides, is transcribed from the same strand as the ribosomal RNA (rRNA) genes and the genes for subunits 1 and 2 of cytochrome *aa*₃. This reading frame shows 65% homology with the yeast gene for the DCCD-binding protein of the mitochondrial ATPase⁷. The other reading frame is 183 nucleotides long and codes for an extremely basic polypeptide (Fig. 1). It is unknown whether this sequence encodes a protein.

Comparison of the amino acid sequence encoded by the former reading frame with the primary structures of DCCD-binding proteins from mitochondria, chloroplasts and bacteria suggests that the *Neurospora* mitochondrial gene codes for a typical DCCD-binding protein⁸ (Fig. 2). This conclusion is based on the following observations: (1) The conservation of glycine at positions 27, 31 and 42, arginine at 45, proline at 47 and alanine at 66. These residues are thought to be indispensable for structure and function, as they have been conserved in all DCCD-binding proteins analysed so far. (2) The conservation of the DCCD-binding glutamic acid at position 65. Aspartic acid is found at the homologous position only in *Escherichia coli*. (3) The absence of histidine and tryptophan. (4) The distribution of the hydrophobic and hydrophilic amino acid residues along the polypeptide chain. Most of the few polar amino acids—all proteins have an extreme low polarity of ~20%—are clustered in the middle of the sequence. This polar segment is flanked on both sides by a long hydrophobic sequence of ~25 residues.

Sebal and co-workers⁸ have demonstrated that in exponentially growing cells of *N. crassa*, the DCCD-binding protein of the mitochondrial ATPase is encoded by a nuclear gene and synthesized in the cytoplasm. Their evidence was based on the finding that cycloheximide, an inhibitor of cytoplasmic protein

Fig. 1 Sequence analysis of the *Neurospora* mitochondrial ATPase proteolipid-like gene. *a*, *Eco*RI fragment 4 of *Neurospora* mitochondrial DNA with the positions of the mitochondrial ATPase proteolipid-like gene (MAL) and the gene for subunit 2 of cytochrome *aa*₃ (COII). Restriction sites: ○, *Hinf*I; ●, *Hae*III; □, *Alu*I (incomplete). The base sequence between the *Alu*I sites, indicated by asterisks, was determined according to Maxam and Gilbert¹⁰. The arrow indicates the direction of transcription. Scale bar, 500 base pairs. *b*, Base sequence of the region containing the mitochondrial ATPase proteolipid-like gene. The sequence of the non-coding strand is numbered starting with A of the start codon. Restriction sites for *Alu*I and *Hinf*I are indicated by solid and broken lines, respectively, above their recognition sequences. The ATPase proteolipid-like gene and that of the unassigned reading frame on the complementary strand are translated into the IUB (International Union of Biochemistry) one-letter amino acid code.

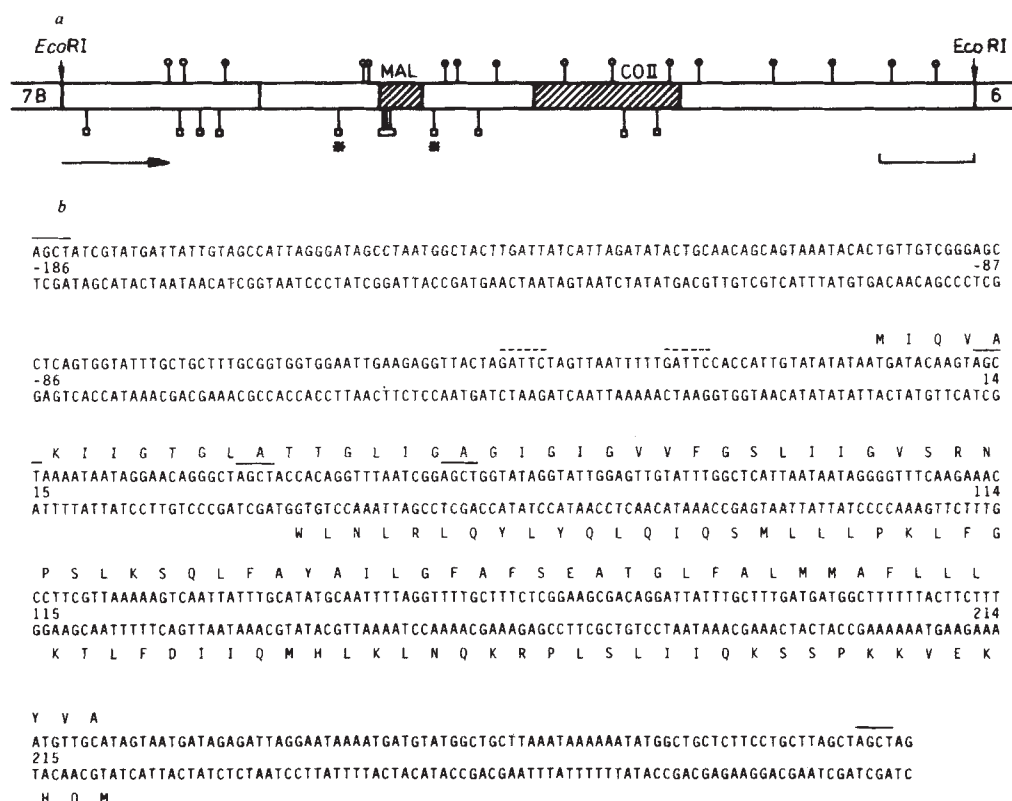


Fig. 2 Amino acid sequences of mitochondrial DCCD-binding proteins from: (1) *N. crassa*, as predicted from the mitochondrial gene; (2) *N. crassa*; (3) *S. cerevisiae*; and (4) bovine heart. The sequences are numbered starting with the first amino acid, tyrosine, of the *Neurospora* protein. Identical amino acid residues in the four sequences are indicated by an asterisk. The amino acid residues which are found to be conserved in all DCCD-binding proteins analysed so far, including those from chloroplasts and bacteria, are boxed. The glutamic acid residue, responsible for the binding of DCCD, is at position 65.

	10	20	30	40	50	60	70	80
1	MIQVAKIIGTGLATTGLIGAGIGIGVVFGLIIIVSRNPSLKSQFAYAILGFAFSEATGLFALMMAFLLLYYA							
2	YSSEIAQAMVEVSKNLGMSAAIGLTGAGIGIGLVFAALLNGVARNPALRGQLFSYAILGFAFVEAIGLFDLMVALMAKFT							
3	MQLVLAAKYIGAGISTIGLLGAGIGIGIAVFAALINGVSRNPSIKDVTFFMAILGFALSEATGLFCLMVSFLLFGV							
4	DIDTAAKFIGAGAAATVGAGSAGIGIGTVFGSLIIIVARNPSLKKQLFSYAILGFALSEAMGLFCLMVAFLILFA							

synthesis, blocks synthesis of the DCCD-binding protein; chloramphenicol, an inhibitor of mitochondrial protein synthesis, had no effect. Furthermore, oligomycin resistance in mutants of *N. crassa* segregates 4:4 at meiosis and not 8:0 as would be expected for a mitochondrial mutation. Sequence studies of the proteolipid from several mutants identified different amino acid substitutions. As no similar mitochondrially synthesized protein has been found in *Neurospora*, we are faced with the enigma of a gene having an as yet unknown function. An obvious possibility is that the mitochondrial gene is silent. Analysis of the transcription products (Fig. 3) demonstrates that the gene lacks a small, unique, mRNA-like transcript, in contrast to the neighbouring gene for subunit 2 of cytochrome *aa*₃. This indicates that the gene possibly does not encode a functional protein. However, we believe that the gene is active because of the striking conservation of the primary structure of the polypeptide. Of course, a very recent inactivation of the gene, as apparently has occurred with the δ -globin gene in Old World monkeys⁹, cannot yet be excluded.

That the translation product of the *Neurospora* mitochondrial gene has gone undetected so far, may be due to its low abundance. However, the DCCD-binding protein occurs as an oligomer of six subunits in each ATPase complex². Therefore, if only one of these subunits were of mitochondrial origin, this

would still constitute some 16% of the total amount of proteolipid of the ATPase complex. Such a high percentage would not have gone undetected in the experiments of Jackl and Sebald⁵. Moreover, our failure to detect a mRNA-like transcript from the gene suggests that in exponentially growing cells of *Neurospora* the gene product, if present, is only a minor mitochondrial translation product. A lower percentage than the 16% mentioned above would indicate functional heterogeneity of the ATPase complex, a phenomenon which, as far as we know, has never been observed. From these considerations we can almost exclude the possibility that the gene is expressed in exponentially growing cells of *Neurospora*. However, expression of the gene might be restricted to another stage of the life cycle of *Neurospora*, for example, to asexual or sexual reproduction. Differential expression of closely related genes is well documented in higher eukaryotes. The occurrence of fetal as opposed to adult haemoglobin chains in mammals¹⁰, and the presence of oocyte-type and somatic cell-type 5S rRNA sequences in *Xenopus*¹¹ are good examples of this phenomenon.

The presence on *Neurospora* mitochondrial DNA of a gene coding for a protein homologous to a nucleus-encoded protein raises the question about the origins of these genes. According to the endosymbiont theory¹² most, if not all, of the nuclear genes encoding organelle-specific proteins have their origin on the endosymbiont genome. In *Neurospora*, the steps that could have led to the occurrence of similar genes on two different genomes are gene duplication, followed by transfer of one of the genes to the nucleus. An evolutionarily recent transfer implies a relatively high degree of homology between the two *Neurospora* proteins. Surprisingly, however, a comparison of the primary structures of the DCCD-binding proteins from mitochondria (Table 1) reveals that the degree of homology is almost the same, and that the similarity between the two *Neurospora* proteins is even lower than the similarities between the mitochondrial gene product from *Neurospora* and the proteins from yeast and bovine heart. This would mean that the divergence of both *Neurospora* genes has occurred in an early period of the evolution of the metazoa, implying that both genes existed in *Neurospora* for a long time. This reinforces our view that the mitochondrial gene is active; inactivation would have resulted in a pseudogene or disappearance of the gene. An alternative explanation for the relatively low homology between the two *Neurospora* proteins is the acquisition of the nuclear gene at a relatively late stage of evolution by uptake of foreign DNA. Such a horizontal gene transfer has similarly been invoked to explain the close homology between vertebrate haemoglobin genes and the leghaemoglobin gene from leguminous plants¹³.

One might ask whether organisms closely related to *Neurospora*, like *Aspergillus* and *Botrydipodia*¹⁴, which also carry the

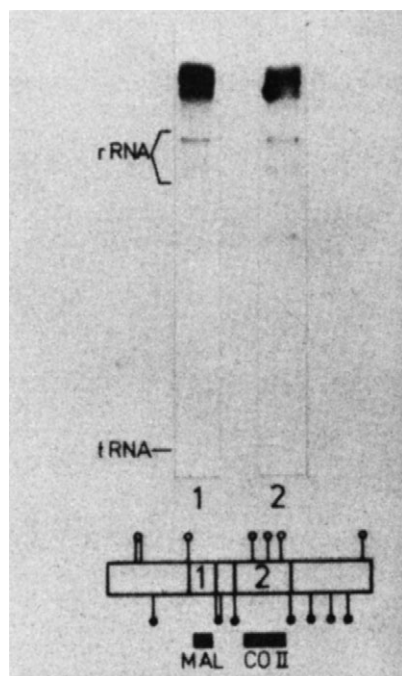


Fig. 3 Transcripts of the mitochondrial ATPase proteolipid-like gene (MAL) and the gene for subunit 2 of cytochrome *aa*₃ (COII). Preparation of blots of mitochondrial RNA on nitrocellulose filters and hybridization conditions were as described by Thomas¹⁷. Probes used for hybridization were *Hae*III and *Hinf*I subfragments (1 and 2) of *Eco*RI fragment 4 of *Neurospora* mitochondrial DNA. ●, ○, The *Hae*III and *Hinf*I restriction sites, respectively. The positions of the rRNAs and tRNAs which contain 3,200, 2,000 and ~80 nucleotides respectively, are indicated in the autoradiograph.

Table 1 Number of identical amino acids in the primary structures of DCCD-binding proteins from mitochondria of *N. crassa*, *S. cerevisiae* and bovine heart

	a	b	c	d
a	74			
b	43	81		
c	49	40	75	
d	46	40	45	76

a, *N. crassa* (primary structure predicted from the mitochondrial gene); b, *N. crassa* (nucleus-encoded); c, bovine heart; d, *S. cerevisiae*.

genes for the DCCD-binding proteins on the nuclear genome, have a mitochondrial gene similar to that of *Neurospora*. If so, nucleotide sequences analysis of both nuclear and mitochondrial genes will not only bear on the origin of these genes, but will also elucidate the function of the mitochondrial gene product.

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UV-induced mutation hotspots occur at DNA damage hotspots

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The frequency of UV-induced mutations at specific nucleotides along the *lacI* gene of *Escherichia coli* varies by as much as 80-fold¹. The spectrum of mutations to amber, ochre, and UGA chain-terminating codons includes five hotspots accounting for over 30% of these UV-induced nonsense mutations¹. The simplest explanation for the hotspot phenomenon is that the mutation frequency reflects the frequency of UV-induced DNA damage at the mutation site. To test this possibility, we measured the distribution, in a 380-base pair (bp) region of the *lacI* gene containing the nonsense mutation hotspots, of UV-induced cyclobutane pyrimidine dimers and pyrimidine-pyrimidine (6-4) photoproducts. The latter (also called the PydC lesion) is a UV-induced DNA lesion which occurs predominantly at TC and CC sequences². The results reported here reveal the presence of UV-induced base damage hotspots which include the nonsense mutation hotspots. There is a linear relationship between base damage incidence and mutation incidence. The results suggest that the UV-induced pyrimidine-pyrimidine (6-4) lesion may be mutagenic.

The distribution of UV-induced DNA damage across the *lacI* gene was determined using methods described previously³. Briefly, fragments of the *lacI* gene labelled at the 3' terminus were irradiated with 254 nm UV light from a germicidal lamp and incubated either with *Micrococcus luteus* UV endonuclease⁴ to incise the DNA at cyclobutane pyrimidine dimers, or with hot alkali to cause strand scissions at sites of (6-4) photoproducts. The pyrimidine-pyrimidine (6-4) lesion is formed in DNA at sites at which cytosine, and much less frequently a thymine,

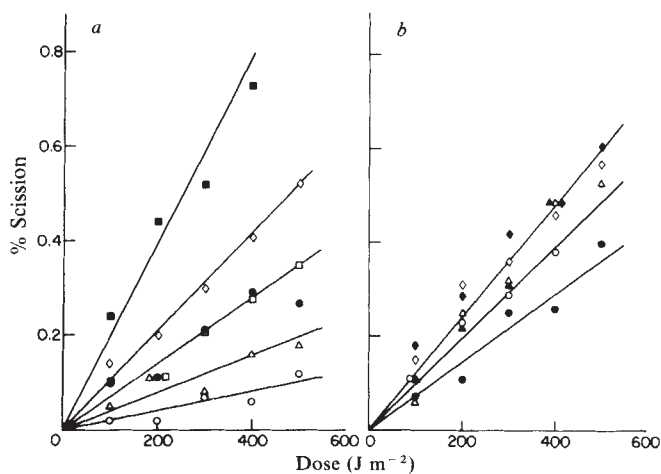


Fig. 1 Dose-response of UV-induced (6-4) lesions and cyclobutane pyrimidine dimers at several sites in the *lacI* gene. Plasmid pMCl containing the *lacI* gene was 3' end-labelled at the bp 565 *Bst*EII or bp 770 *Sau*3AI site, re-cut with *Hinc*II or *Sau*96I, and appropriate fragments isolated spanning the region from bp 380 to 760 of plus and minus *lacI* strands. DNA was irradiated with 500 J m⁻² of UV light (254 nm), incubated with piperidine to cause strand scission at (6-4) sites, or with *M. luteus* UV endonuclease to incise at cyclobutane-type pyrimidine dimers, and analysed on 8% urea-containing polyacrylamide gels with G + A and C + T Maxam-Gilbert sequencing reactions as markers. Gels were then autoradiographed. Bands were cut from the polyacrylamide gels and analysed by Cerenkov counting, the data were corrected for multiple cuts within the same DNA fragment as described elsewhere¹⁷, and the percentage of initial molecules carrying scissions at a particular site was computed. The background was subtracted. *a*, (6-4) lesions at CC dinucleotides 627 (○), 671 (△) and 676 (□), and at TC dinucleotides 665 (●), 684 (◇) and 689 (■). *b*, Cyclobutane pyrimidine dimers at CT dinucleotides 661 (●) and 691 (△), and at TT dinucleotides 637 (○), 655 (◇), 709 (▲) and 673 (◆).

is located 3' to a pyrimidine base² and is the precursor to the UV-induced Thy(6-4)Pyo, Cyd(6-4)Pyo, and Thy(6-4)m⁵Pyo photoproducts described by Wang and co-workers (refs 5, 6 and W. A. Franklin, K. M. Lo and W.A.H., unpublished observations). The products of reaction of TC, CC and TT sequences are observed in irradiated DNA, whereas that of CT is not (W. A. Franklin, K. M. Lo and W.A.H., unpublished observations).

After treatment of the UV-irradiated DNA with either the UV-specific endonuclease or hot alkali, the products were analysed on denaturing high-resolution polyacrylamide gels. The precise site and extent of strand scission, and therefore the site and incidence of the lesions, can be determined using this method. The sites of cyclobutane dimer damage and (6-4) products can be identified separately². The percentage of initial molecules damaged at a particular site was determined by measuring the radioactivity in each cleavage product, correcting for multiple lesions within the same DNA fragment.

The extent of formation of both the cyclobutane pyrimidine dimers and the (6-4) lesions was proportional to the incident dose over the dose range 100–500 J m⁻² (Fig. 1). The slope of the dose-response curve varied widely from site to site for both cyclobutane pyrimidine dimers and (6-4) photoproducts. The linearity of the dose-response between 100 J m⁻² (the approximate dose at which mutants were generated¹) and 500 J m⁻², permitted measurement of relative damage frequencies at the higher dose. We have shown previously that the distribution of base damage in DNA irradiated with UV light *in vitro* is the same as that of DNA irradiated *in vivo*². Therefore, the distribution of UV-induced damage sites measured here should reflect the distribution of DNA damage in the irradiated cells.

The frequencies of (6-4) photoproducts and pyrimidine dimer lesions at specific sites within the *lacI* gene are listed in Table 1,

Table 1 Distribution of UV-induced base damage in the *lacI* gene

Dinucleotide	Site*	Sequence	(6-4) Product (%)	Dimer (%)	Mutants	Substitution	Nonsense site no.
TC	396	TC	0.17	0.21			
	457	TTCC	0.21	0.89			
	469	TTTCTTT	0.05	0.43			
	477	TCTCT	0.00	0.38			
	479	TCTCT	0.09	0.39			
	495	TC	0.42	0.16			
	510	TTCT	0.23	-			
	512	TCCC	0.35	-			
	640	TCTC	0.18	0.33			
	642	TCTC	0.52	0.20			
	646	CTC	0.41	0.18			
	652	TC	0.86	0.82	43	C→T	O24
	658	TTT	0.49	0.36	80	C→T	A23
	665	TC	0.21	0.16			
	672	TTCC	0.60	0.86	1	C→A	O25
	678	TTCC	0.83	-	4	C→A	O26
	689	CTCC	0.57	0.48	39	C→T	U6
	698	TCC	0.07	0.03			
	706	TTTTC	0.98	0.91	60	C→T	O27
	728	TTT	1.66	-			
	732	CCCTC	1.08	-	0	C→A	A25
CC	738	TC	0.27	0.21			
	418	CC*	0.01	0.35	7	C→T	A15
	426	CC	0.06	0.18			
	444	CCT	0.00	0.17			
	458	TTCC	0.09	0.27			
	484	CC	0.16	0.25	20	C→T	A16
	491	CCC	0.27	0.24			
	492	CCC	0.43	0.34			
	513	TCCC	0.24	-			
	514	TCCC	0.27	-			
	608	CC	0.35	-0.05			
	623	CC	0.03	0.02			
	627	CC	0.04	0.00			
	631	CC	0.16	0.07			
	671	TTCC	0.11	0.26			
	676	CCC	0.35	-			
	688	CTCC	0.15	0.53	9	C→T	A24
	693	CC	0.18	-0.01			
	699	TCC	0.08	0.09			
	702	CC	0.07	0.35			
CT	714	CC	0.13	0.33			
	432	CT	-	0.46			
	441	CT	-	0.19			
	445	CCT	0.00	0.73			
	450	CT	-	0.74			
	470	TTTCTT	-	0.66			
	478	TCTCT	0.00	0.38			
	480	TCTCT	0.09	0.39			
	533	CT	-	0.18			
	584	CT	0.14	0.42			
	597	CTT	-	0.40			
	624	CT	-	0.13			
	628	CT	-	0.17			
	641	TCTC	-	0.22			
	645	CTC	-	0.45			
	661	CT	-0.04	0.18			
	668	CT	0.00	0.46			
	685	CT	0.04	0.22			
	690	CTCC	0.09	0.66			
TT	725	CT	0.02	0.04			
	429	TT	-	1.20			
	456	TTCC	-	1.05			
	467	TTTCTT	-	0.80			
	468	TTTCTT	-	1.87			
	471	TTTCTT	-	1.53			
	504	TT	-	0.07			
	507	TTTTC	0.03	0.32			
	508	TTTTC	0.03	0.87			
	509	TTTTC	-	1.64			
	596	CTT	-	1.00			
	636	TTT	0.08	2.08	1	T→A	Y4
	637	TTT	-	0.86			
	651	TT	0.02	1.00			
	655	TTT	0.03	1.43			
	656	TTT	-0.07	0.59			
	657	TTT	-	0.66			
	673	TTCC	0.04	0.38			
	703	TTTTC	-	0.13			
	704	TTTTC	-	1.22			
	705	TTTTC	-	2.26			

Incidence of (6-4) lesions and cyclobutane pyrimidine dimers at specific sites was determined as described in Fig. 1 legend. The number of nonsense mutants isolated for a particular dinucleotide, with the 3' base being the mutating base, was obtained from Table 3 of Coulondre and Miller¹. Ochre mutations were normalized for comparison with amber mutations, but UGA mutations cannot be accurately normalized¹⁶. Base damage hotspots include dinucleotides 492, 495, 608, 642, 652, 658, 672, 678, 689, 706, 728 and 732. * First base of dinucleotide. † 5-Methylcytosine.

together with the frequency of UV-induced nonsense mutations at the same sites, as determined by Coulondre and Miller¹.

Regarding the distribution of UV-induced base damage: (1) The yield of T<gtT dimers at almost all potential T<gtT sites is high compared with that of other lesions. (2) The incidence of T<gtC, C<gtT and C<gtC dimers varies five-fold from site to site. At the high dose range, the incidence of these dimers equals that of T<gtT dimers. (3) The incidence of (6-4) photoproducts varies dramatically (10-15-fold) from site to site. The incidence of T<gtC lesions is in general greater than that of C<gtC lesions. (We propose that the (6-4) photoproduct type lesions be abbreviated as T<gtC, C<gtC, etc. This abbreviation symbolizes the single bond linking the two adjacent nucleotides.) At very high doses ($5,000 \text{ J m}^{-2}$) some T<gtT lesions are discernible. The stereochemical basis for the preferential formation of the (6-4) lesion in DNA at TC and CC sequences, compared with TT and CT sites, will be explained elsewhere. At most sites the (6-4) lesion is several-fold less frequent than the pyrimidine dimer at the same site. (4) At some sites the frequency of the (6-4) photoproduct is equal to or greater than that of the dimer; at such sites the frequency of both the (6-4) lesion and the dimer is increased to levels typical of T<gtT dimers. These sites are hotspots for UV-induced base damage. (5) The reactivity of TC and CC sequences for both (6-4) photoproduct and cyclobutane dimers at potential nonsense sites (but not all sites) depends on the number of A·T base pairs located 5' to, and including, the site (Fig. 2a). Reactivity is also increased at TC and CC sequences at the 3' end of pyrimidine tracts. (6) At a CC sequence at which the 3' cytosine is methylated, the frequency of cyclobutane pyrimidine dimers equals that at similar unmethylated CC sequences. However, the (6-4) photoproduct is not detected at that site.

Correlation of the distribution of DNA damage with the site-specific frequency of UV-induced chain-terminating codons reveals no correlation between the frequency of nonsense mutations and the frequency of T<gtT dimers, even though mutations from TT sequences to chain-terminating codons are permitted by the selection system. Furthermore, none of the UV-induced nonsense mutation hotspots occur at TT sequences, despite the observation that T<gtT dimers constitute the majority of UV-induced lesions.

There are two possible explanations for this. As only transversions can give rise to chain-termination codons at TT sites, the low incidence of mutation at such sites may reflect a low probability of UV-induced transversion events at damage sites. Alternatively, the low frequency of nonsense mutations at TT sites may reflect the low yield of (6-4) photoproducts at such sequences.

The latter possibility may be correct, as there is an excellent correlation between the frequency of nonsense mutations and the frequency of UV-induced damage at TC and CC sequences. The greater the UV-induced damage at such sequences, the greater the observed mutation frequency (Fig. 2c). The correlation is rather better for the (6-4) photoproducts than for the cyclobutane dimer. For example, even though the UV-induced dimer frequency at position 688 (a CC sequence) is the highest measured for such sites, the induced mutation frequency and extent of (6-4) product formation are among the lowest measured. At other sites of C→T transition, similar levels of dimer formation give rise to far more mutations, for example, sites 689, 658 and 484. At site 418, the UV-induced mutation frequency is also much lower than expected based on the high level of dimer formation. At this site the 3' cytosine of the CC sequence is methylated, and the (6-4) product is not detected. The appreciable mutation frequency that occurs at both sites 418 and 688 is typical of the minimum number of transition mutations occurring at dipyrimidine sites.

At most sites, the extent of dimer formation is greater than that of the (6-4) lesion, and the UV-induced mutation frequency is relatively low. However, at the nonsense mutation hotspots, not only is the T<gtC or C<gtC dimer frequency greater than at

other sites, but the (6-4) photoproduct frequency equals or exceeds that of the dimer (Table 1).

The slope of the correlation between the extent of base damage and nonsense mutation frequency is greatest for sites at which the 3' cytosine undergoes a transition to a thymine.

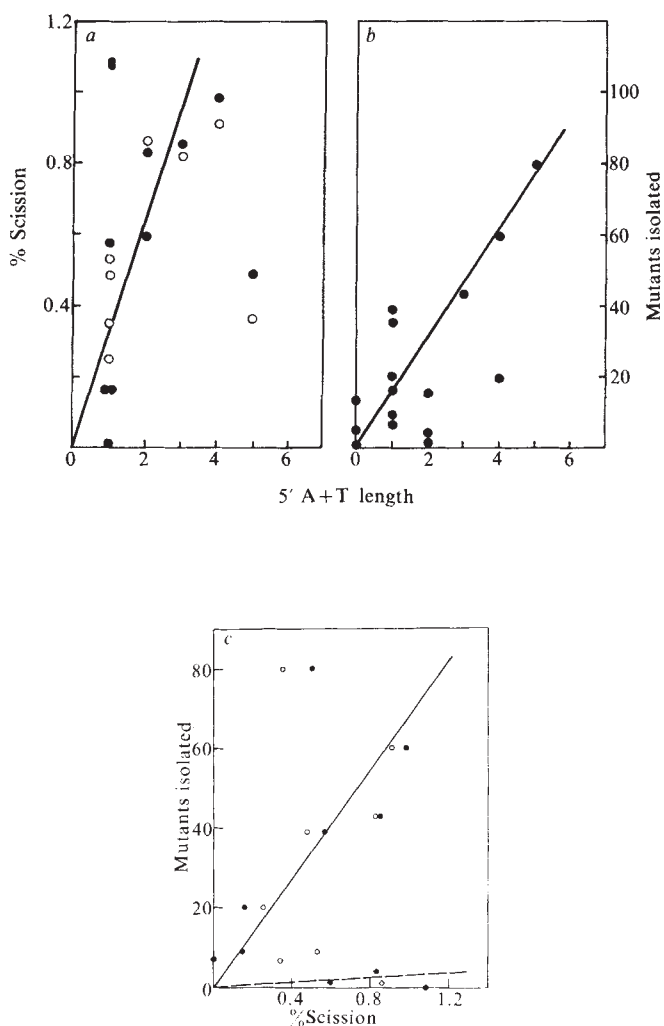


Fig. 2 Relationships of site-specific nonsense mutations, DNA damage, and 5' A+T composition. The number of nonsense mutants isolated for a particular base pair was obtained from Table 3 of Coulondre and Miller¹, as described in Table 1 legend. DNA damage incidence was measured as per cent scission, as described in Fig. 1 legend. A+T length was scored as the number of contiguous A·T base pairs 5' to the PyPy dinucleotide, including any A·T in the PyPy sequence. *a*, Dependence of UV-induced base damage on 5' A+T composition: (6-4) lesions (●) and cyclobutane pyrimidine dimers (○) at nonsense mutation sites. *b*, Dependence of UV-induced nonsense mutations on 5' A+T composition: nonsense mutations at sites of C→T substitution (●) are shown. (Nonsense mutations at sites of other substitutions are not dependent on A+T composition.) *c*, Dependence of site-specific incidence of nonsense mutation on site-specific base damage incidence: (6-4) lesions (●, ◆) and cyclobutane pyrimidine dimers (○, ◇) at C→T substitution sites (—) and at C→A substitution sites (---). Site A23 is an apparent exception to the correlations of Fig. 2a,c.

All transversions involving pyrimidines occur at low frequency, regardless of the extent of formation of either the cyclobutane dimer or the (6-4) lesion at such sites. This observation implies that the mechanisms for mutation are biased for transition rather than for transversion mutations at UV-induced lesions. A preference for C→T transition mutations at potential dimer sites has been reported by others using systems with a sufficiently large collection of target sequences⁷⁻¹¹.

The data suggest that (6-4) photoproducts are mutagenic but do not exclude the possibility that cyclobutane dimers are also mutagenic. The selection for nonsense mutation excludes transition mutations from thymine to cytosine, as well as simple shifts in reading frame. If these are the major mutagenic events associated with cyclobutane pyrimidine dimers, then these would have been missed in this analysis. However the data do exclude the possibility that cyclobutane dimers lead to a high frequency of transversion mutations. On the other hand, the observation that some nonsense mutations do arise at TT and CT sequences does not prove that cyclobutane dimers are mutagenic, as other infrequent lesions at these sequences, such as the T/T product, may account for these events.

The experiments presented here permit the distribution of the UV-induced nonsense mutations in the *lacI* gene to be interpreted in terms of the distribution of UV-induced damage. Hotspots for UV-induced nonsense mutations arise at hotspots for base damage, provided that amber, ochre, or UGA codons can be derived by a transition mutation. To some extent, the frequency of occurrence of DNA damage can in turn be predicted from the primary sequence of the DNA: TC or CC sequences located 3' to A+T-rich regions have been shown to be hotspots for UV-induced damage, provided that the 3' cytosine at the site is unmethylated. A direct correlation between the 5' A+T content and nonsense mutation frequency at transition sites is plotted in Fig. 2b. Consequently, the relative frequency of UV-induced nonsense mutations can be predicted from the DNA sequence alone.

Miller and Schmeissner¹² also report the presence of hotspots for UV-induced missense mutations in the *lacI* gene. The selection for missense mutations is not constrained by the same rules that apply to the selection of nonsense mutations. The generality of the observations regarding the correlation between UV-induced base damage and mutation reported here can be tested by determining the sequence of missense hotspot mutations. If the (6-4) products constitute an important class of premutagenic lesion, a strong bias for mutation at TC sequences compared with CT sequences should be observed.

We are aware of the extensive literature suggesting that cyclobutane pyrimidine dimers are the major mutagenic lesion induced by UV light^{13,14}. For the most part, such experiments correlate the physiology of the cyclobutane pyrimidine dimers with the mutagenic event. These experiments do not exclude the possibility that another lesion having similar properties may be the site of a major mutagenic event¹³⁻¹⁵. The present study suggests that the (6-4) photoproducts may be mutagenic and indicates the need for further studies of this lesion.

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Rescuing the *in vitro* function of a globin pseudogene promoter

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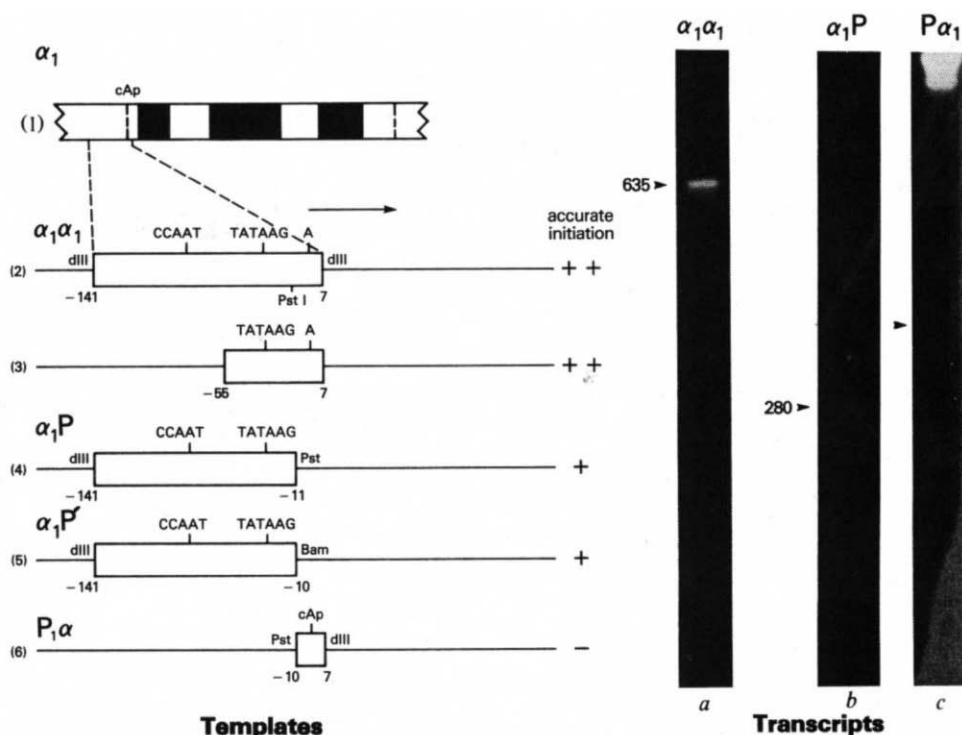
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The mouse globin gene loci are collections of active genes and inactive pseudogenes¹⁻⁴. Not only are the globin pseudogenes α_3 and α_4 not expressed in globin-producing cells³, but RNA polymerase II does not transcribe these templates *in vitro* in conditions in which transcription of the active genes (for example, α_1) is initiated accurately^{1,5}. To localize the globin gene promoter and to determine whether the pseudogenes are promoter mutants whose function can be rescued by replacing mutant sequences with the analogous wild-type DNA, we have now analysed the transcriptional activity of several truncated and hybrid templates constructed from active α_1 and inactive α_4 genes. Our results indicate that a 44-base pair (bp) stretch of DNA (spanning nucleotides -55 to -11) is sufficient to direct RNA polymerase II to initiate transcription accurately *in vitro*. However, transcription efficiency is increased if the stretch of α_1 -globin DNA at the capping/initiation site (from -10 to +7) is included as part of the promoter segment. Analysis of hybrid promoters constructed from the active and pseudogenes, α_1 and α_4 , demonstrated that replacement of the pseudogene's capping/initiation site with that of the active gene (nucleotides -9 to +7) restores the pseudogene promoter function *in vitro*. In addition, transcription studies with two other hybrid promoters indicate that the pseudogene's capping/initiation site is not simply a negative control element, but that RNA polymerase II may actually recognize and interact with a segment of DNA up to 60 nucleotides long.

One approach to localizing globin gene promoter regions is to replace stretches of cloned globin genomic DNA with foreign DNA (for example plasmid pBR322)⁶ and determine whether the remaining globin sequences can direct RNA polymerase II to initiate transcription accurately *in vitro*. We have used the mouse globin genes and a HeLa cell cytoplasmic extract for this purpose¹. For example, a 148-bp α_1 -globin DNA fragment extending from nucleotide -141 to nucleotide +7 (where nucleotide +1 is the initiation site) ligated into pBR322 contains sufficient information to be recognized accurately by RNA polymerase II¹. As shown schematically in Fig. 1 (template 2), this 148-bp DNA contains not only the initiation site but also TATAAG hexanucleotide identified in almost all RNA polymerase II-regulated genes analysed and the CCAAT pentanucleotide found in many of the same genes (see, for example, refs 7-9). Transcription of a second plasmid (Fig. 1, template 3) indicates that sequences upstream from -55, including the CCAAT pentanucleotide, are not required for accurate initiation of globin gene transcription *in vitro*¹⁰. This finding contrasts with studies using a viral thymidine kinase promoter

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Fig. 1 Schematic maps and *in vitro* transcription studies of plasmids tested for promoter function. Template 1 shows a portion of the clone containing the entire α_1 -globin gene plus flanking sequences (2.1 kb) ligated into pBR322¹. Template 2 ($\alpha_1\alpha_1$) consists of the *Hind*III-linked 148-bp fragment of α_1 -globin DNA (extending from -141 to +7) ligated into the *Hind*III site of pBR322. Template 3 contains the 62-bp *Bst*NI/*Hin*FI fragment of α_1 -globin DNA (extending from -55 to +7, including the TATAAG and capping sequences) that had been *Eco*RI-linked and ligated to the *Eco*RI site of pBR322. Both plasmids 2 and 3 are oriented with respect to pBR322 such that the *Bam*HI site at pBR322 map position 375 is 345 bp to the right of the rightmost *Hind*III boundary or 375 bp to the right of the right-most *Eco*RI boundary, respectively. The last three templates show the construction of plasmids which the capping site has been separated from sequences upstream from -10. By restricting plasmid $\alpha_1\alpha_1$ at *Pst*I (position -11) and at *Hind*III, we constructed plasmids α_1P (template 4) and $P\alpha_1$ (template 6) (that is, each globin DNA fragment was joined to the 3.6-kb *Hind*III/*Pst*I fragment of pBR322), as indicated schematically. For plasmid α_1P , the *Bam*HI site is 345 bp to the left of the *Hind*III boundary, and the relevant *Dde*I site is 290 bp to the right of the *Pst*I boundary. For plasmid $P\alpha_1$, the *Bam*HI site is 345 bp to the right of the *Hind*III boundary. Plasmid 5 was constructed by restricting the globin sequences of plasmid $\alpha_1\alpha_1$ at the *Ava*II site (position -10) and at *Hind*III; the larger *Hind*III/*Ava*II globin-containing fragment was ligated to the 4-kb *Hind*III/*Bam*HI fragment pBR322; thus, the *Hinc*II site at map position 650 is 275 bp to the right of the *Bam*HI boundary. (In this construction we found that an *Ava*II end will ligate directly to a *Bam*HI end.) ³²P-labelled RNAs were synthesized and analysed by acrylamide-urea gel electrophoresis¹. Each transcription reaction contained $\sim 2 \times 10^6$ cell equivalents of HeLa cell 2-100 extract and 2 μ g of the following template: lane a, *Hinc*II-cleaved $\alpha_1\alpha_1$ plasmid DNA; lane b, *Dde*I-cleaved α_1P plasmid DNA (there are eight *Dde*I restriction sites in this plasmid); lane c, *Bam*HI-cleaved $P\alpha_1$ plasmid DNA. The sizes, in bases, of the RNA polymerase II-dependent transcripts are indicated to the left of lanes a and b. The arrowhead to the left of lane c indicates the predicted location of the 360-bp specific transcript that would have been synthesized if the α_1 -globin fragment contained within the $P\alpha_1$ plasmid were functional promoter. To compare the amounts of RNA polymerase II-dependent RNA synthesized in these reactions, the autoradiographs were scanned by a densitometer and standardized with respect to the amount of 'end-to-end' transcription of restriction fragments, a level which is relatively invariant between reactions.



recognition site and the frog oocyte nucleus as a transcription system¹¹, where the segment between nucleotides -100 and -40 is essential for accurate transcription. This difference may reflect the fact that the DNA template is incorporated into a chromatin-like structure within the frog oocyte before transcription¹¹.

To determine how the TATAAG hexanucleotide and the initiation/capping site influence transcription *in vitro*, we separated those sequences by restricting the 148-base active α_1 -globin sequence at position -11 so as to construct the two distinct plasmids shown in Fig. 1, templates 4 and 6. Transcription of the α_1P plasmid (spanning -141 to -11) yielded a discrete RNA polymerase II-dependent species (Fig. 1, lane b) that was initiated 25-30 bases downstream from the TATAAG sequence that is, analogous to positions -5 to +1), as determined by *S*₁ nuclease mapping analysis. In contrast, transcriptional analysis of $P\alpha_1$ (spanning -10 to +7) resulted in no RNA polymerase II-dependent RNA synthesis (Fig. 1, lane c). Thus, accurate initiation of transcription of the active α_1 -globin gene promoter could occur in the absence of the proper initiation site. However, the replacement of α_1 RNA between nucleotides

-10 and +7 with pBR322 DNA markedly decreased the efficiency of transcription initiation (Fig. 1 (compare lanes a and b)). This result was also found for a second plasmid in which a different portion of pBR322 was substituted for the normal α_1 initiation site (Fig. 1, template 5, data not shown).

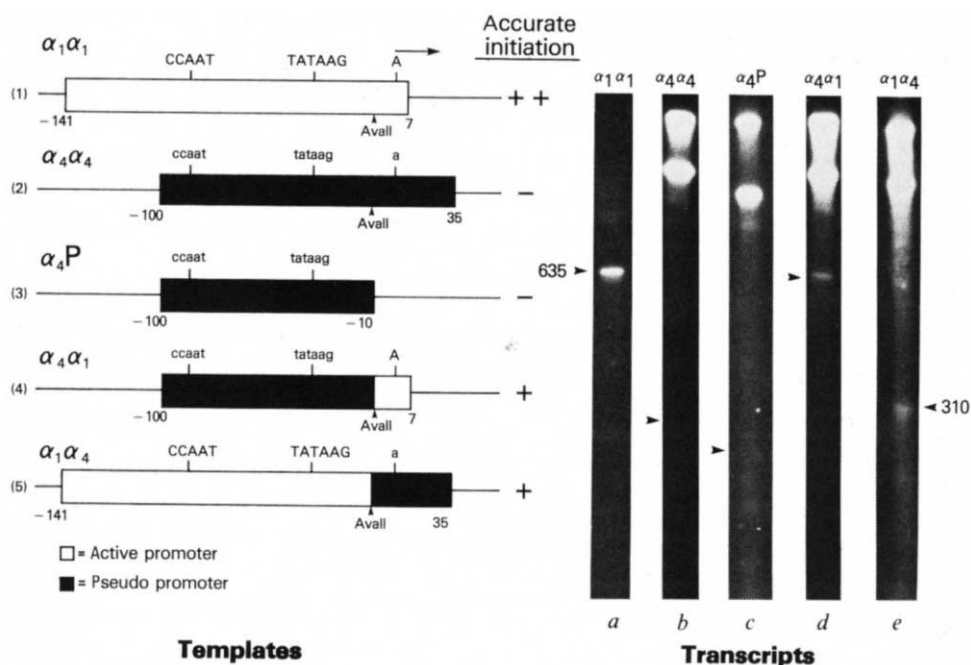
We conclude from these studies that the globin gene promoter need extend, at most, from nucleotides -55 to -11 to direct RNA polymerase to initiate *in vitro* transcription accurately. In addition, the sequences between nucleotides -10 and +7 apparently modulate the rate of initiation. A similar form of analysis on several RNA polymerase II-dependent genes by several other laboratories as well as our own has also implicated the TATAAG₆ hexanucleotide and the immediately surrounding sequences as being necessary for *in vitro* transcription. For example, the promoters of the adenovirus late gene and the rabbit β -globin gene have been localized to stretches of 21 nucleotides (-32 to -12)¹² and 15 nucleotides (-34 to -20)¹³, respectively.

As mentioned above, neither pseudogene α_3 nor α_4 is transcribed in our HeLa cell cytoplasmic extract¹. As the promoter region of the active α_1 gene has been delimited to a 44-bp

Fig. 2 Nucleotide sequence comparison of the active α_1 -globin gene and pseudogenes α_3 and α_4 . Nucleotides are numbered such that position +1 is the transcription initiation site; positive numbers denote transcribed nucleotides. Vertical lines indicate nucleotides shared by α_1 and either α_3 or α_4 , respectively. The CCAAT pentanucleotide, CCAAG pentanucleotide, TATAAG hexanucleotide and the capping site are each enclosed in boxes.



Fig. 3 Transcription analysis of hybrid promoters constructed from active and pseudogene DNA segments. The autoradiogram in lane *a* displays the RNA polymerase II-dependent transcript synthesized from the *HincII*-cleaved active α_1 -promoter-containing plasmid (α_1 -globin sequences extending from -141 to +7, as shown schematically on the left, template 1). Lane *b* displays the transcripts synthesized from the *HincII*-cleaved plasmid containing the inactive α_4 promoter. The plasmid used consists of the α_4 DNA fragment extending from -100 to +35 ligated into the large *HindIII*/*BamHI* fragment of pBR322 (template 2); therefore, the predicted size of an RNA polymerase II-dependent transcript would be 310 bases. Lane *c* displays the transcripts from the *HincII*-cleaved α_4 P plasmid (shown schematically on the left, template 3). This plasmid, derived from plasmid $\alpha_4\alpha_4$, consists of the 90-bp *HindIII*/*AvaII* α_4 DNA fragment ligated into the 2.9-kb *HindIII*/*BamHI* fragment of pBR327. The arrowhead to the left of the lane indicates the predicted location of the 265-base specific transcript that would have been synthesized if this α_4 -globin DNA fragment contained a functional promoter. Lane *d* displays the transcripts synthesized from the hybrid promoter $\alpha_4\alpha_1$ shown schematically on the left (template 4). This plasmid consists of a *HindIII* segment containing α_4 sequences from -100 to -10 joined to α_1 sequences from -9 to +7 (via the *AvaII* site) ligated into the *HindIII* site of pBR322 in the same orientation as plasmid $\alpha_1\alpha_1$. Thus, the RNA polymerase II-dependent transcript generated from the *HincII*-cleaved hybrid-promoter-containing plasmid is the same size (635 bases) as that from the *HincII*-cleaved α_1 -promoter-containing plasmid. Lane *e* displays the transcripts synthesized from the *HincII*-restricted hybrid promoter $\alpha_1\alpha_4$ shown schematically on the left (template 5). The RNA polymerase II-dependent transcript of 310 bases is indicated by the arrowhead. This plasmid consists of a *HindIII*/*Sau3A* segment containing α_1 sequences from -141 to -10 joined to α_4 sequences from -9 to +35 (via the *AvaII* site) ligated to the 2.9-kb *HindIII*/*BamHI* pBR327 fragment. Thus, the *HincII* site is 275 bases to the right of the *BamHI* boundary. 32 P-labelled RNAs were synthesized and analysed as described in ref. 1.



region (plus an 18-bp 'modulator'), we can ask whether the pseudogenes' sequences within that analogous region are responsible for their apparent inactivity. The nucleotide sequences of α_1 (ref. 14), α_3 (ref. 3) and α_4 (Y. Nishioaka, unpublished) are shown in Fig. 2. For α_3 , the T at nucleotide position -28 (one of the most highly conserved nucleotides within the TATAAG hexanucleotide) has been altered to a G. Whether this nucleotide change is sufficient to abolish promoter function is unknown, but having a T at position -28 (instead of a G) is crucial for *in vitro* expression of the conalbumin gene¹⁵.

Comparison of the pseudogene α_4 and the active gene α_1 indicates that those two sequences are identical at and immediately surrounding the TATAAG hexanucleotide (nucleotides -34 to -21). However, they differ dramatically at the initiation site where the active α_1 sequence of AGACACT (nucleotides -4 to +3) differs from the pseudogene α_4 sequence of GCAAATT in four positions (base changes underlined). If these nucleotide substitutions are sufficient to block initiation, the activity of the pseudogene's α_4 promoter could potentially be 'rescued' by replacing the α_4 sequences downstream from -10 with the active α_1 initiation/capping site. The construction of such a hybrid promoter containing α_4 sequences from -100 to -10 and α_1 sequences from -9 to +7 (plasmid $\alpha_4\alpha_1$) is shown schematically in Fig. 3, template 4. Transcription of *HincII*-restricted $\alpha_4\alpha_1$ yielded an RNA polymerase II-specific transcript of 635 bases (Fig. 3, lane *d*). Thus, accurate initiation of transcription was restored, albeit at a lower efficiency, suggesting that this region influences transcription and—among other possibilities—that the α_4 initiation/capping site might function as a negative control element to block initiation.

To test this latter possibility, we constructed two other promoter-containing plasmids in which the presence or absence of the pseudogene's capping/initiation segment might elucidate its function. In one case, the α_1 promoter was altered such that the active α_1 initiation site (that is, sequences downstream from -10) was replaced with that of the pseudogene α_4 ; plasmid $\alpha_1\alpha_4$ is shown in Fig. 3 (template 5). Transcription of the

HincII-restricted $\alpha_1\alpha_4$ plasmid yielded a discrete RNA polymerase II-dependent species of 310 bases (Fig. 3, lane *e*), indicating that the 4-bp substitutions at nucleotides -4, -3 and +2 in α_4 were not sufficient to block promoter function completely. The efficiency of transcription, however, was reduced approximately fivefold.

To determine if simple removal of the α_4 initiation site from the pseudogene α_4 promoter would relieve inhibition, a plasmid was constructed in which the α_4 segment of DNA spanning nucleotides -100 and -10 was ligated into pBR327¹⁶ (plasmid α_4 P is shown in Fig. 3, template 3) to form a 'promoter' analogous to α_1 P (Fig. 1, template 4). Transcription of this DNA yielded no specific RNA (Fig. 3, lane *c*) indicating that pseudogene α_4 sequences upstream from nucleotide -10 could not direct RNA polymerase II to initiate even though the active α_1 and inactive α_4 genes are identical between -34 and -21 and highly homologous elsewhere in the region.

All these results suggest that the interaction between RNA polymerase II and globin gene promoter is not limited to merely a few nucleotides surrounding the 'TATAAG' hexanucleotide; this is not a surprising result because RNA polymerase is a large (>500,000 molecular weight), multimeric protein. In fact, it is likely that RNA polymerase II specifically recognizes nucleotides near or at the initiation site in order to initiate transcription, and this expectation is fulfilled by our finding that the pseudogene α_4 promoter is restored by replacing its initiation/capping site with that of the adult α_1 -globin gene. The capping site is not simply a negative control element blocking initiation in any environment, because the $\alpha_1\alpha_4$ hybrid is active. Further, the initiation site apparently is not only feature responsible for the inactivity of pseudogene α_4 because its removal (for example, template α_4 P) is not sufficient to restore promoter activity. Consequently, RNA polymerase II recognizes more than just the TATAAG sequence *in vitro*. Thus the globin gene promoter apparently comprises a target over a region as large as 62 bp (-55 to +7) with which RNA polymerase II interacts.

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Multiple H4 histone mRNAs of HeLa cells are encoded in different genes

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We have previously reported the presence of at least two distinct mRNAs coding for histone H4 in HeLaS3 cells^{1,2}. In this communication we report the resolution of at least seven different histone H4 mRNAs, each of which codes for histone H4. The presence of different mRNAs coding for the same protein could be explained either by the presence of different genes that have diverged without changing the encoded amino acid sequence (there are 20-40 copies of the histone genes per haploid genome in human cells³), or by differential processing of the same primary transcript. By hybridizing a series of cloned genomic human H4 histone sequences to the histone H4 mRNAs and digesting with S₁ nuclease, we have shown that different H4 histone mRNA species are protected from nuclease digestion by different H4 histone genes, suggesting that at least three of the HeLa H4 histone mRNAs are the products of distinct genes.

In vitro ³²P-labelled 7-11S polysomal RNA from S phase HeLa cells was electrophoresed under denaturing conditions in a 6% (w/v) acrylamide-8.3M urea gel at 55-60°C (Fig. 1a), and the three RNA bands (A, B, and C) in the H4 region were excised from the gel, and the RNA eluted. When each of these H4 histone mRNA fractions was re-electrophoresed on the same type of denaturing gel, single bands were obtained and there was only a small amount of cross-contamination between species (data not shown). These labelled RNA fractions were identified as H4 histone mRNAs by *in vitro* translation⁴⁻⁶, and from hybridization with a chicken H4 histone gene-containing clone (data not shown). Each of the three RNA fractions yielded several bands when electrophoresed under nondenaturing conditions (Fig. 1b). Each of the RNAs directed the translation of H4 histone in a cell-free system (data not shown), and the

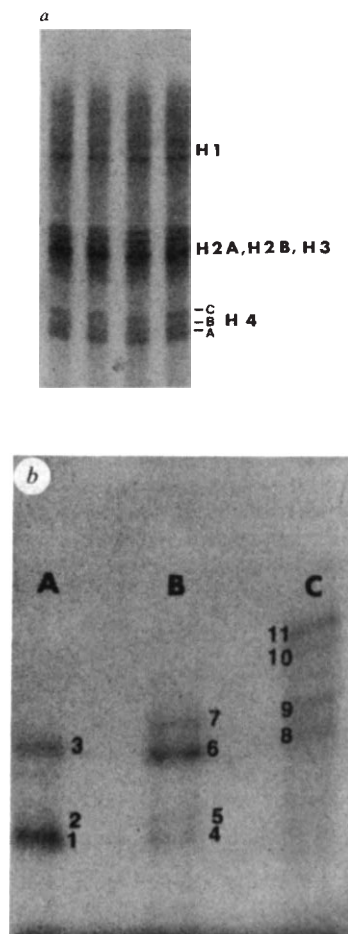


Fig. 1 *a*, HeLa S3 cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum. Unlabelled total polysomal RNA was isolated from 5×10^9 S phase cells^{37,38} synchronized by a single 2 mM thymidine block³⁹. ³²P-labelled RNA was prepared from one litre of S phase HeLa cells synchronized by two cycles of 2 mM thymidine block³⁹. Following release from the second thymidine block the cells were incubated in phosphate-free growth medium buffered with 30 mM HEPES containing 10% dialysed calf serum and 50 mCi of ³²P (2×10^6 cells ml⁻¹) at 37°C for 4.5-5 hours. Cells were collected and lysed in 10 mM Tris-HCl pH 7.5, 13 mM MgCl₂, 10 mM vanadyl ribonucleoside, as described³⁸. Unlabelled 5-18S RNA was isolated using similar procedures, mixed with ³²P-labelled RNA and ethanol precipitated. The pellet was dissolved in 8.3 M urea, 5 mM EDTA (pH 8.0), heated to 100°C and quick cooled before loading on a 6% (w/v) polyacrylamide, 8.3 M urea gel buffered with 50 mM Tris-borate, 1 mM EDTA (TBE)⁴¹. The gel was run for 7 hours at 20 watts, which gives a surface temperature of 55-60°C. After autoradiography, the bands labelled A, B, and C were excised and eluted electrophoretically⁴². The sizes of the H4 mRNAs, as determined by electrophoresis in MeHgOH-3% (w/v) agarose gels using *HpaII/PstI* restriction fragments of ϕ X174 DNA as markers, are 364, 394 and 439 bases (R. Rickles, personal communication). *b*, RNA eluted from the gel shown in *a* was ethanol-precipitated and the dried pellet was dissolved in 4 M urea, 2.5 mM EDTA (pH 8.0), heated to 100°C for 2 min and quick cooled on ice. The samples were loaded on a non-denaturing 'strand separation' gel⁴¹ containing 5.9% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 50 mM TBE and 0.2% (w/v) SDS. Electrophoresis was at 15 watts (400 V) for 8 hours.

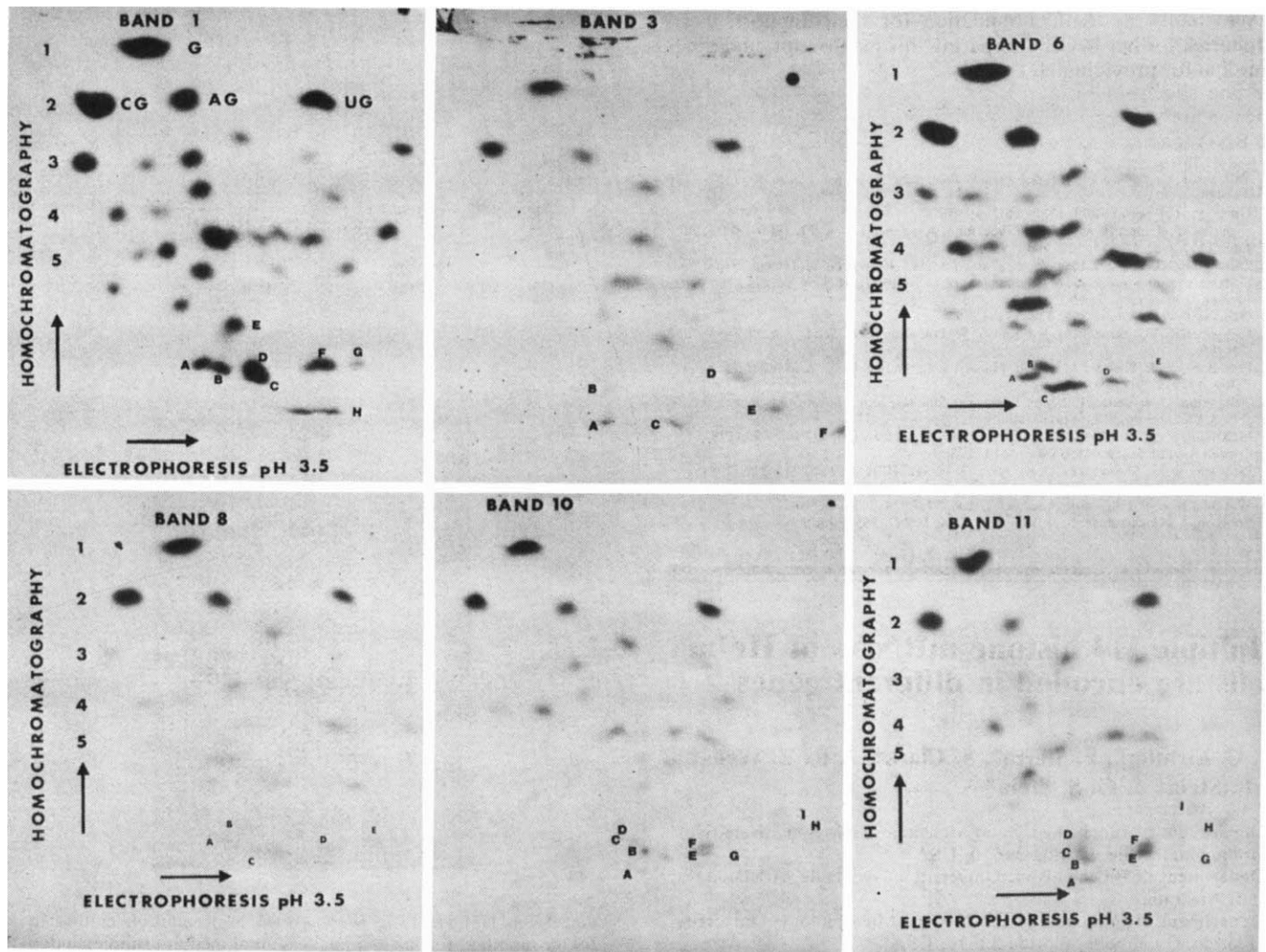


Fig. 2 Two-dimensional T_1 ribonuclease fingerprint analysis of H4 histone mRNAs. ^{32}P -labelled RNAs extracted from a gel similar to that shown in Fig. 1b were digested with T_1 ribonuclease and analysed according to the method of Volkaert *et al.*⁷. Briefly, T_1 ribonuclease-digested RNAs were electrophoresed on cellulose acetate strips at pH 3.5 in 7 M urea, transferred to PEI-cellulose thin layer chromatography plates, and developed with homochromatography mix B⁷ at 70 °C. The fractionated oligonucleotides were detected by autoradiography and the labelled spots were eluted, subjected to ribonuclease A digestion, and analysed according to Barrell⁸.

amount of H4 histone synthesized correlated with the amount of radioactivity present in the RNA bands. When the RNAs in each of the 11 bands in Fig. 1b were eluted from the gel, denatured by heating at 100 °C in 4M urea and re-electrophoresed in another nondenaturing gel, each migrated as a single species with the same relative mobility as in the original gel (data not shown). These results suggest that the multiple H4 mRNA bands seen in the nondenaturing gel system represent several different H4 histone mRNA species, rather than conformational isomers of a single species.

To examine the extent of differences among the various H4 histone mRNAs, we analysed the oligonucleotides generated by T_1 ribonuclease digestion of histone H4 mRNA bands A1, A3, B6, C8, C10 and C11 from Fig. 1b using the two-dimensional fingerprint technique of Volkaert *et al.*⁷ (Fig. 2). The largest oligonucleotides indicated in each panel of Fig. 2 were further analysed by ribonuclease A digestion⁸ (data not shown). The results of this analysis establish that the histone H4 RNAs A1, A3 and B6 are completely distinct species, with only one of the large T_1 oligonucleotides shared by A1 and B6. C8 was identical to B6 and probably represents cross-contamination of H4C by H4B. C10 and C11 shared seven of nine T_1 oligonucleotides, and were both different from A1, A3 and B6.

We have isolated several λ Charon 4A recombinant clones, containing human histone gene sequences⁹ (designated λHHG). The seven clones thus far characterized fall into three distinct types of arrangements, based on their restriction site patterns and the location of protein coding regions. We have used these clones in hybridisation experiments to elucidate the origins of the multiple histone H4 mRNAs. Clones representing each of the three classes were used: λHHG 41 represents one class, λHHG 39 a second class, and λHHG 6 and λHHG 17 a third class.

The assignment of histone H4 mRNAs to their respective genomic coding sequences was based on resistance of the mRNA-DNA hybrids to S_1 nuclease, which under the conditions used in our studies will hydrolyse a single mismatched base pair¹⁰. Our experiments involved annealing mixtures of electrophoretically purified ^{32}P -labelled H4 histone mRNAs with the histone gene-containing recombinant phage DNA in 80% formamide, and digesting with S_1 nuclease according to the procedure of Berk and Sharp¹¹ as modified by Haegeman *et al.*¹². The RNA species which remain S_1 nuclease resistant are those which are complementary to the DNA over their full length. To identify the RNA species that were protected from S_1 nuclease digestion by the cloned DNA, the S_1 digested

samples were divided and half of each sample was electrophoresed in a denaturing gel containing urea, and half in a non-denaturing gel. RNA bands eluted from a gel similar to the one shown in Fig. 1a were electrophoresed in adjacent lanes as markers. An 8.3 M urea—6% (w/v) acrylamide gel of the RNA species which are protected from S_1 nuclease digestion by hybridization with λ HHG 17, λ HHG 39 or λ HHG 41, along with marker histone mRNAs H4A, H4B and H4C, is shown in Fig. 3a. λ HHG 17 protects RNAs comigrating with two H4 histone RNA species, H4A and H4B, which is consistent with other evidence that this clone contains two H4 histone genes⁹. λ HHG 39 protects one RNA that comigrates with H4B, as does λ HHG 41. The results of fractionation of the S_1 nuclease resistant RNA samples in a nondenaturing gel are shown in Fig. 3b. λ HHG 17 protects two H4 mRNAs (lane 2), one a subspecies of H4A (band A3, Fig. 1b), the other a minor species of H4B (band B7, Fig. 1b). The independent isolate λ HHG 6, apparently identical to λ HHG 17, also protects subspecies H4A3 and H4B7; Fig. 3b lanes 4 and 6 shows that the two *Eco*RI fragments of λ HHG 6 each protect a different subspecies. The H4 histone mRNAs complementary to λ HHG 39 (lane 1) and λ HHG 41 (lane 8) both comigrate with the major H4B subspecies, H4B6 (see Fig. 1b). In the case of λ HHG 41, we have additionally been able to show that the H4 histone mRNA protected from S_1 nuclease digestion has a two-dimensional T_1 ribonuclease fingerprint identical to that of the H4B6 mRNA (not shown).

The results presented here suggest that there are at least three types of human H4 histone genes, each encoding a different H4 histone mRNA. Two of the types of H4 histone genes are each present in the genomic clones λ HHG 6 and λ HHG 17. Although λ HHG 39 and λ HHG 41 both appear to protect the H4B6 mRNA, it is possible that there may be minor variations in the H4 genes of these two clones. Although λ HHG 17 and λ HHG 41 reproducibly protected a large fraction of their complementary H4 mRNA species, λ HHG 39 always yielded a weak mRNA band, suggesting that λ HHG 39 may be complementary to a less abundant H4 mRNA, which co-migrates with H4B6 in both the denaturing and non-denaturing gel systems used here. Although we have distinguished at least seven different H4 histone mRNA species, we have been able to assign only three of them to the corresponding genes. These results imply that there may be four or more types of human H4 histone genes in addition to those we have characterized.

Several examples of multiple mRNAs coding for similar or identical proteins have been described. Some, such as mouse dihydrofolate reductase¹³ and α -amylase¹⁴ mRNAs are produced from a single gene by variable transcription or RNA processing. Others, including the actin mRNAs of humans^{15,16} and *Dictyostelium*^{17,18} are encoded in multiple, non-identical gene copies.

Histone genes and histone mRNAs have been most extensively studied in sea urchins. Sea urchins have two major classes

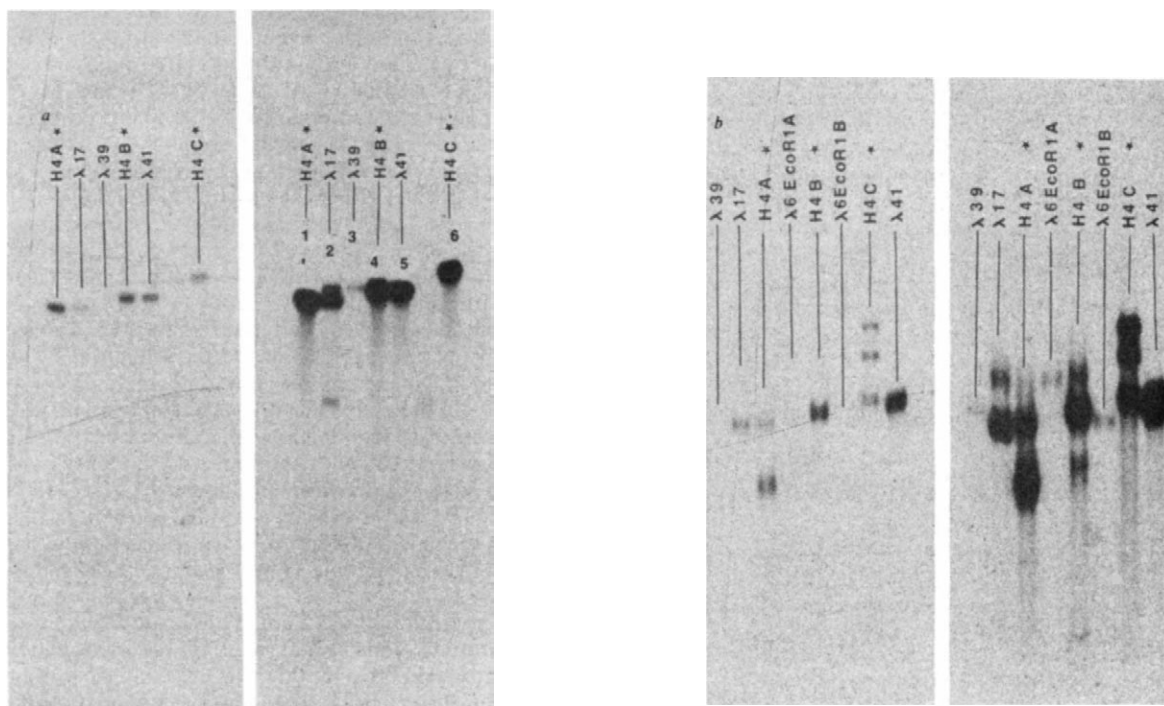


Fig. 3 Identification of H4 histone mRNA species which are S_1 nuclease resistant after hybridization to histone DNA clones. 10–20 μ g of λ HHG recombinant DNAs were mixed with 500–2,000 c.p.m. of 32 P-labelled total H4 histone mRNA isolated from a gel similar to the one shown in Fig. 1 and precipitated with ethanol in a 1.5 ml microfuge tube. The pellet was drained thoroughly and dissolved in 20 μ l redistilled but not deionized formamide (Bethesda Research Laboratories). 4 μ l of 2 M NaCl, 0.4 M PIPES (pH 6.4), 5 mM EDTA were added, and the tube was placed at 80 °C for 5 min. Hybridization was at 50 °C for 5 hours. After hybridization, 225 μ l of ice cold 0.25 M NaCl, 25 mM Na acetate (pH 4.4), 0.45 mM ZnSO₄ were added and the tube was mixed thoroughly and immediately placed on ice. 110 units of S_1 nuclease (Sigma) in 5 μ l were then added, and the sample incubated at 37 °C for 30 min, 20 μ l of 10% (w/v) SDS, 20 μ l 0.2 M EDTA (pH 8.0), 20 μ g tRNA, 200 μ l of phenol, and 200 μ l chloroform/isoamyl alcohol (24:1) were added, and the mixture was vortexed, centrifuged, and the supernatant was ethanol precipitated. The dried pellet was dissolved in 8.3 M urea, 5 mM EDTA, heated to 100 °C for 2 min and electrophoresed on an acrylamide gel. **a**, S_1 nuclease resistant samples electrophoresed in an 8.3 M urea gel. Lane 1, H4A histone mRNA. Lane 2, λ HHG 17 protected RNA. Lane 3, λ HHG 39 protected RNA. Lane 4, H4B histone mRNA. Lane 5, λ HHG 41 protected RNA. Lane 6, H4C histone mRNA. Light (left panel) and dark (right panel) exposures of the autoradiograms are shown. **b**, S_1 nuclease resistant samples electrophoresed in a nondenaturing gel. Lane 1, λ HHG 39 protected RNA. Lane 2, λ HHG 17 protected RNA. Lane 3, H4A histone mRNA. Lane 4, λ HHG 6 *Eco*RI fragment A protected RNA. Lane 5, H4B histone mRNA. Lane 6, λ HHG 6 *Eco*RI fragment B protected RNA. Lane 7, H4C histone mRNA. Lane 8, λ HHG 41 protected RNA. Light (left panel) and dark (right panel) exposures of the autoradiograms are shown.

of H4 histone mRNAs, which are preferentially expressed at different stages of development^{19,20}. The early H4 mRNAs of *Lytechinus pictus* have three very similar forms²¹, the genes for which are repeated several hundred times and are arranged in tandemly repeated clusters^{19,20}. Although they encode exactly the same protein the late and early H4 mRNAs are very divergent²²⁻²⁷. The late sea urchin histone genes are repeated 25–50-fold²² but they have only recently been cloned²⁸ and details of their organization have not yet been published.

The histone genes of *Drosophila*²⁹ and *Notophthalmus*³⁰ are similar to the early sea urchin genes in their highly repetitive nature and ordered structure, while the histone genes of *Xenopus*³¹⁻³³, chicken^{34,35} and human^{9,36} are more analogous to the late sea urchin genes in being reiterated 10–50-fold and in not being highly ordered. It is not known however if higher eukaryotes have classes of histone genes that are functionally equivalent to the early and late histone genes of sea urchins. The ability to resolve a separate H4 histone mRNA for each human H4 histone gene provides us with a unique opportunity to address this question. Because H4 histone proteins do not have variant forms, the study of variant H4 mRNAs is the most direct method for detecting the expression of different H4 histone genes. Variant histone H4 mRNAs have not been detected in organisms other than sea urchins and humans.

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A model for base overlap in RNA

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Duplexed RNA in the solid state conforms to the A-family of structures with 3'-endo sugars and bases arranged outside the helix axis in a spiral staircase arrangement¹⁻⁴. Recent experiments⁵⁻¹¹ suggest that parameters of RNA and DNA helices depend on the sequence of bases in the chain. Furthermore, the average conformation of these flexible molecules may differ between the solution and solid states. The NMR chemical shifts of the base protons in ten oligo-RNA duplexes^{5,12-18} have been determined. We have now adjusted the helix winding angle, W , between adjacent base pairs (see Fig. 1a) to fit these data. There is extensive overlap of the hydrophobic surfaces of the bases at the proposed angles. These overlaps depend on the arrangement of purines and pyrimidines along the chain and offer a mechanism for recognition of specific sequences by enzymes.

The analysis forbids ranges in W shown as bars in Fig. 2. Figure 2a indicates that a purine next to a purine on the same strand favours $W = 50^\circ \pm 5^\circ$, while Fig. 2c shows that a 5'Py-Pu3' arrangement leads to $W = 45^\circ \pm 10^\circ$ except for the C-G:C-G stacking interaction, which is likely to prefer $W \leq 35^\circ$. Figure 2b illustrates that the current data do not restrict W as tightly for 5'Pu-Py3' orientations; note the large blank areas. However, $W = 20^\circ \pm 5^\circ$ is satisfactory in all cases. The internal consistency of the analysis is remarkable. None of the proposed winding angles is the same as the 30° value for A'-RNA (RNA-12) derived from X-ray diffraction of RNA fibres¹.

Base-stacking, rather than hydrogen-bonding, confers stability to nucleic acid helices. Note the extensive contact between the hydrophobic surfaces of the bases at the proposed winding angles (see Fig. 1d-f). This is a compelling molecular explanation of our observations.

Simple rules of base overlap lead naturally to ideas regarding the enzymatic recognition of specific sequences by their shape. RNA and DNA sequences should be analysed for regularities in Pu-Pu, Pu-Py, Py-Pu, or nearest-neighbour arrangement. For example, splice junctions in unprocessed messenger RNA, control regions of genes and repeated sequences in DNA may contain such regularities. The unusual C-G:C-G orientation may provide a special signal as it is represented in eukaryotic mRNA much less often than predicted on a random basis¹⁹.

The NMR spectrometer views an average conformation of the nucleic acid. The microsecond- to nanosecond-scale events known to occur in the motion of duplexed DNA²⁰ might correspond to oscillations in base overlap, the hydrophobic surfaces of the bases sliding over one another.

The base protons of an isolated mononucleotide are shielded by an amount, $\Delta\delta$, when the monomer is incorporated into a duplexed oligomer. The $\Delta\delta$ values of the three or four protons on each base pair are fitted by adjusting the winding angles on either side of that pair, and are estimated from isoshielding contours including ring-current and local atomic anisotropies²¹. Figure 3 illustrates the process for several protons in duplexed AAGCUU. The first four proton 'maps' exclude certain combinations of winding angles W_{12} and W_{23} , that is, the solid regions where theory and experiment disagree by more than 0.25 parts per million (p.p.m.). The four maps are then correlated in Fig. 3e, the solid areas occupying most of this map. The process is repeated for the next base pair, yielding a new correlation map in Fig. 3f relating W_{23} and W_{34} . Figure 3e and

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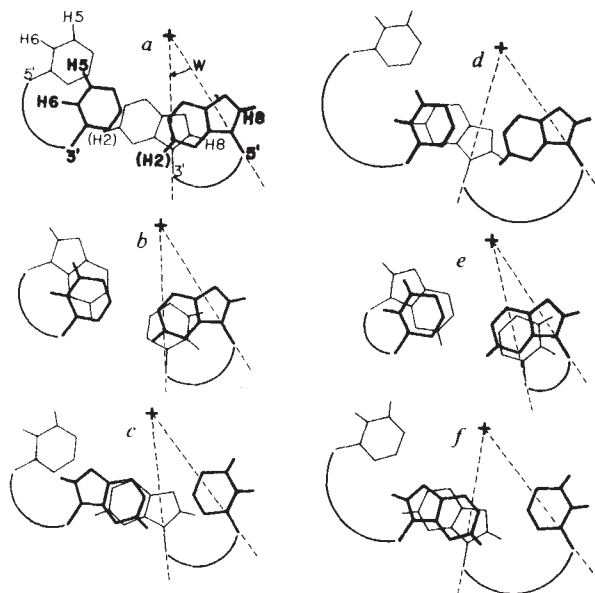


Fig. 1 Base-pair overlaps in RNA viewed along the helix axis. Pairs drawn in heavy outline lie nearest the observer. Views *a* (Pu-Pu), *b* (Pu-Py) and *c* (Py-Pu) show overlaps in A'-RNA with winding angles $W = 30^\circ$. Corresponding views *d* ($W = 50^\circ$), *e* ($W = 20^\circ$) and *f* ($W = 45^\circ$) show overlaps suggested by the NMR analysis. The circular arcs denote the covalent ribose-phosphate linkage between bases. *a-c* Were traced from Fig. 2b of Hukins *et al.*¹, which are projections of the base pairs on to a plane approximately perpendicular to the helix axis. The glycosidic nitrogen atoms vary as much as 7% in distance from the helix axis due to the projection chosen and the tilt and twist of bases in a pair.

f are then compared to exclude more values of W_{23} (see Fig. 3 legend for further details).

Figure 2 compiles the results of this analysis, the solid bars indicating W values excluded when theory and experiment disagree by ≥ 0.25 p.p.m.; these bars are extended by the open bars when we use a criterion of ≤ 0.15 p.p.m. as an acceptable fit.

We have refrained from making monomer corrections similar to those suggested by Mitra *et al.*^{22,23}, although a mononucleotide certainly undergoes a change in its conformational equilibrium when incorporated in a duplexed oligomer. This probably includes base-specific changes⁵ in glycosidic torsion angle, χ . Pullman and associates have calculated profiles of $\Delta\delta$ against χ for various protons, but warn against literal interpretation of these theoretical $\Delta\delta$ values²⁴. Mitra *et al.* based their 'corrections' on these numbers and measurements of the nuclear Overhauser enhancement (NOE) of protons in guanosine monophosphates²⁵. Only a small number of microgeometries

in the array of *syn* and *anti* states contribute to the NOE effect. Mitra *et al.* took these particular microstates (one in the *syn* range and a linear average of the two in the *anti* range) to amend their $\Delta\delta$ calculations. With a more realistic view of the distribution and populations of states in the monomers, one should obtain very different corrections.

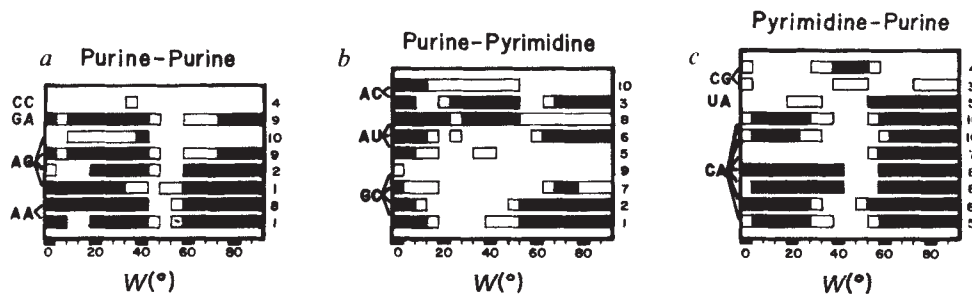
Figure 2 implies that these and other corrections for alterations in tilt, twist and second-neighbour shielding must be of the order of 0.25 p.p.m. or less. (1) There is a remarkable pattern of agreement among the 27 independent determinations of W . (2) The pattern is consistent with a simple molecular explanation. (3) Changing the criterion of acceptable fit from 0.15 p.p.m. to 0.25 p.p.m. has little effect on this pattern except to widen the zones of acceptable fit. (4) Monomer corrections due to changes in *syn/anti* equilibrium have little effect on the pyrimidine protons. (5) Figure 2 emphasizes that the analysis only excludes certain winding angles. Suppose we leave certain protons out of the analysis which we suspect might have large monomer corrections. The effect will be to widen the acceptable ranges— W values that were acceptable previously will still be allowed. (6) The key point is that the excluded angles could not change in a concerted fashion if a large monomer correction was made. For example, the A-G:C-U stacking interaction is represented in four duplexes. In each, the A·U pair is in a different environment and its $\Delta\delta$ values must be fitted by simultaneous adjustment of both adjacent winding angles. The result of a large monomer correction on any of the A·U protons is a change in most of the bars in Fig. 2, but this change would be unlikely to produce a new W_{AG} consistent among the four duplexes. Similar arguments hold for the other stacking interactions.

The shielding of most protons is very sensitive to changes in W with A-family models. Gradients in the shielding fields of the bases are large for protons located above or below a ring; Fig. 1 makes it clear that changes in W carry many of the protons across the surface of neighbouring rings. The allowed regions in Fig. 2 change little within a reasonable range of error estimates because there are substantial gradients in the shielding fields for some protons at the favoured winding angles.

Thus far we have had little success in fitting spectra of DNA oligomer duplexes using B-family configurations. There are three problems. First, rotation around the helix axis moves most protons through small gradients in the shielding fields compared with rotations in the A-form. Second, there is a paucity of shielding data on DNA oligomer duplexes where resonances have been uniquely assigned. Third, the helix axis intersects the base pairs such that optimum base overlap occurs only near $W = 0$ or 180° , both unacceptable alternatives. Because it is impossible to make strong base overlaps at acceptable winding angles in the B-form, it is possible that this structure is more easily deformed than A-structures upon interaction with other biomolecules.

Uncertainties remain in these results. Some will be solved easily using a computer representation of accurate atomic

Fig. 2 The excluded winding angles at two levels of the quality of fit, ± 0.25 p.p.m. (solid bars) and ± 0.15 p.p.m. (open plus solid bars). Bars are plotted in discrete 5° intervals whenever correlations as in Fig. 3 indicate a forbidden winding angle. Dinucleotides indicated to the left of each panel denote the bases along one strand of the 10 unique stacking interactions: A-A:U-U, A-C:G-U, A-G:C-U, A-U:A-U, C-A:U-G, C-C:G-G, C-G:C-G, G-A:U-C, G-C:G-C, U-A:U-A. Numbers at the right of each bar denote the molecule used in that analysis: 1¹³, (AAGCU)₂; 2⁵, (AGCU)₂; 3⁵, (ACGU)₂; 4¹², (CCGG)₂, ambiguous assignment of protons; 5¹⁷, (CAUAG)₂; 6^{15,16}, (CAUG)₂; 7¹⁸, (UGCA)₂; 8¹⁷, CAUAG:CAUUG, correlation of W_{34} with W_{45} produces no area of fit within a ± 0.15 p.p.m. criterion, therefore ± 0.20 p.p.m. was used to determine the non-excluded regions on this correlation map; 9¹⁴, GAGC:GCUC; 10¹⁷, CAGUG:CACUG.



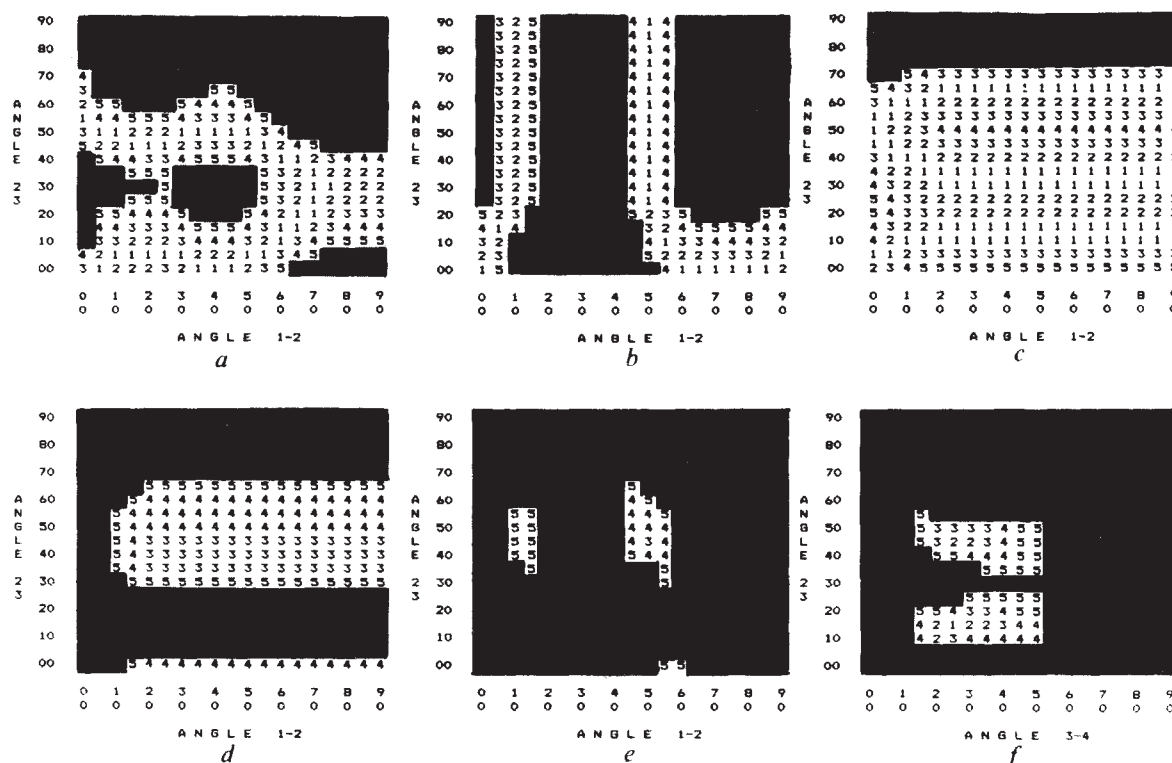


Fig. 3 Two-dimensional winding angle maps for A(2)H2 (a), A(2)H8 (b), U(2)H5 (c) and U(2)H6 (d) of AAGCUU. Each of these maps exclude certain combinations of W_{12} and W_{23} . Correlation maps for these four protons (e), and G(3)H8, C(3)H5, C(3)H6 (f) are also shown. Quality of fit, $|Q|$, is defined by $0 < |Q| \leq 0.05$ p.p.m. and is represented by a 1 in the maps, $0.05 < |Q| \leq 0.10$ by a 2, $0.10 < |Q| \leq 0.15$ by a 3, $0.15 < |Q| \leq 0.20$ by a 4, $0.20 < |Q| \leq 0.25$ by a 5 and $|Q| > 0.25$ is represented by a black area. The array elements on the correlation maps represent the worst fit at that point on any of the correlated single proton maps, in accord with the above numbering scheme. The arrays are constructed by comparing the measured $\Delta\delta$ with that computed from isoshielding contours at all combinations of the two winding angles indicated in the panels of the figure. The computations are performed by rotating projections of base pairs onto a plane perpendicular to the helix axis at 5° intervals in W over the $0-90^\circ$ range. Resonances from base pairs at the helix ends are not used in the fitting process as they are influenced by rapid fraying and possible end-to-end aggregation^{5,27}; thus, the first and last W values in a chain are determined solely by resonances on the penultimate base pairs.

coordinates and a more complete description of the shielding fields²²⁻²⁴. The A'-RNA model tilts the bases by 10° with respect to the helix axis and has an 8° propeller-twist between bases in a pair. There are likely to be $\pm 5^\circ$ deviations from these values dependent on composition. However, such variations in tilt and twist will not move the protons through large gradients in magnetic shielding. We consider these to be second-order perturbations and below the resolution of NMR at the present level of understanding of the spatial dependence of the shielding fields.

There is some question of the applicability of these results to native systems because the ends of oligomer duplexes are unconstrained; W may vary more freely than in high polymers. However, natural RNA is folded with short helical segments joined by relatively unconstrained single-stranded regions. Thus, oligomers are probably suitable models.

We conclude that the overlap of the bases contributes substantially to the helix form in duplexed RNA oligomers. The proposed winding angles offer a simple explanation of the NMR spectra of a large collection of RNA molecules, are insensitive to reasonable error estimates and may represent a part of the mechanism by which enzymes recognize specific sequences.

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Note added in proof: L.-S. Kan (personal communication) calculated $\Delta\delta$ for CCAAGCTTGG in the A'-geometry including tilt, twist and second-neighbour effects. Our calculations at $W = 30^\circ$ agree within 0.10 ± 0.05 p.p.m. for the 15 protons we can compare.

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MATTERS ARISING

Supposed squirrel monkey affinities of the late Oligocene *Dolichocebus gaimanensis*

THE Patagonian late Oligocene long-headed monkey, *Dolichocebus gaimanensis* Kraglievich, was categorized by Rosenberger¹ as a "member of the *Saimiri* lineage, which thus becomes the oldest generic lineage known for the primates, dating from about 25 Myr ago". This was based on the following "shared derived characters".

First, the presence of an interorbital fenestra in *Dolichocebus* and in squirrel monkeys, genus *Saimiri*. With the skull of *Dolichocebus* in hand, the subovate interorbital perforation (Fig. 1) appears to me an artefact of preparation. The right side of the opening differs from that of the left. Its greatest diameters, about 6.9 mm × 5 mm, are about 0.2 mm longer and wider than those of the left, with neither side precisely in line with the other. The position of the opening relative to the optic foramen is too far anterior for a natural fenestra and cuts through the area where the turbinal bones are normally located. Remnants of mineralized bone on the inferior border are too gross to have formed part of the normally sharp single laminate edge of a natural vacuity. Preserved bone on the superior border is also too thick to have been included either in



Fig. 1 *a*, Thick-walled perforated interorbital septum of *Dolichocebus gaimanensis* (Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN) 14128) right side. *b*, Thin-walled translucent naturally fenestrated septum in *Saimiri sciureus* (Field Museum of Natural History (FMNH) 18553). *c*, Stereoscopic view of left *Dolichocebus* orbit, slightly foreshortened because the angle of exposure to the camera exaggerates the real difference in outline between the perforations of the left and right sides; arrows used for orientation point to the same break between the nasal and frontal bones on the left and right sides. *d*, Dorsal view of *Saimiri* (FMNH 46174); *e*, dorsal view of *Dolichocebus* to the scale shown; actual greatest cranial length = 70.1 in *Saimiri*, 69.1 in *Dolichocebus*.

a fenestral border or a former translucent area. A centripetal projection of its borders following the natural contour of the septum, would close the hole completely.

A large, natural, knife-edged, translucent-bordered interorbital fenestra, such

as is consistently present in *Saimiri* (Fig. 1), is shared with all living members of the order Lagomorpha (pikas, rabbits, hares). It is also present but much smaller in some species of Rodentia, Carnivora and Artiodactyla². Among primates, a

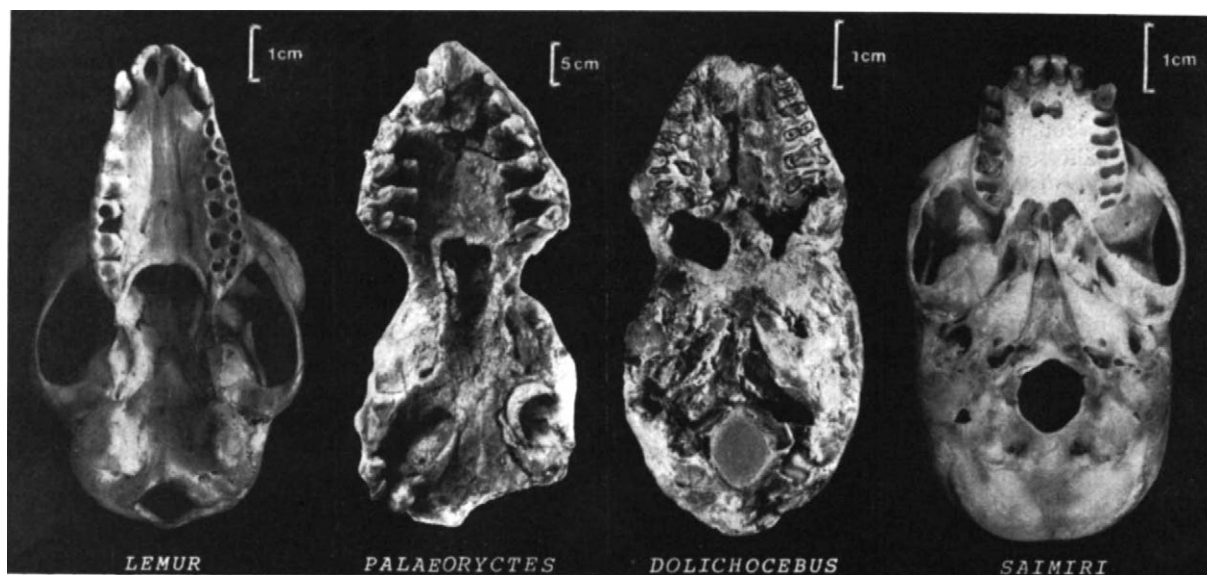


Fig. 2 Basicranial surface of *Lemur macaco* (FMNH 81543); *Palaeoryctes puericensis*, a Palaeocene proteutherian or insectivore (*sensu lato*) (American Museum of Natural History 15923)⁴; *Dolichocebus gaimanensis* (MACN 14128); and *Saimiri sciureus* (FMNH 18553). Left cheek teeth of *Lemur* and *Saimiri* removed for comparison at the alveolar plane with truncated teeth of *Dolichocebus*. The dental arcade of *Dolichocebus* resembles that of the unrelated *Palaeoryctes*, and the outline of its cheek teeth remnants are nearer the zalambdomorphic-dilambdomorphic crown pattern of *Palaeoryctes* and other Insectivora (*sensu lato*) than the eutheriomorphic pattern of Primates. The *Dolichocebus* first molar crown base lacks any sign of a hypocone.

small natural perforation has been observed in individuals of *Tarsius*, *Macaca*, *Cercopithecus* and *Papio*^{2,3}. Obviously, the trait by itself is no indicator of phylogenetic relationship. Should the interorbital aperture of the Oligocene *Dolichocebus* skull still prove to be natural and as large as represented by Rosenberger¹, it is unlikely that it would have persisted through 25 Myr of descent to reappear unchanged in the grossly different *Saimiri* skull.

The second shared characteristic mentioned by Rosenberger was the "great anteroposterior length of the frontal interorbital process, unique to these species [*Dolichocebus gaimanensis* and *Saimiri sciureus*]"¹. However, measurements are not given. Actually, the interorbital bones of *Dolichocebus* are shattered, the sutures untraceable, with size and osseous relationships of the frontal interorbital process absolutely indeterminable.

Narrow nasals, another apparently "shared" characteristic¹, are, in fact, shared with many primate species, while "Reduced petromastoids" is, again, a bald assertion.

A prominent and impressive feature of Rosenberger's article is his "reconstruction" of the right side of the *Dolichocebus* skull. Cranial contour, angles and curves of the reconstruction, however, are not those of the fossil, which actually appears undistorted in right lateral view¹. Also, size of anatomical details such as auditory meatus, right orbit (compared with intact left of fossil) and teeth is not that of corresponding features of *Dolichocebus*. Comparative size of cheek teeth is $pm^2 < pm^3 < pm^4$ in *Dolichocebus* (Fig. 2) but $pm^2 > pm^3 \leq pm^4$ in the reconstruction¹. Incisors and their alveoli are entirely lost in *Dolichocebus* but the dental arcade must have been V-shaped (Fig. 2). Contour of the projecting *Saimiri*-like incisors shown in the reconstruction is that of a U-shaped arcade.

The remainder of Rosenberger's article deals with "several indications that the masticatory system of *Dolichocebus* was rather lightly built". The "indications" include a description of the form and function of masticatory muscles recreated from traces of bones, absent bones and a barely discernible right temporal line. The not completely erupted right canine tooth of the unappreciated dental system, however, indicates a young animal. With sexually dimorphic monkeys like *Cebus apella* in mind, the likelihood of a heavy masticatory system in a mature male *Dolichocebus* cannot be discounted³.

Passed over by Rosenberger in his discussion of the masticatory system is the *Dolichocebus* dentition represented by stubs of canines and cheek teeth (Fig. 2). The sheared surfaces are measurable and reflect the occlusal pattern. Most striking is the antero-posterior narrowness of the teeth and their progressive transverse expansion from canine (2.5 mm) to first

molar (6.3 mm) or >2.5 times, and from first premolar (2.9 mm) to first molar or nearly 2.2 times.

The expanding gradient is characteristic of zalambdomorphic-dilambdomorphic tooth rows as in 'insectivores' *Nesophontes*, *Potomogale*³ or *Palaeoryctes* (Fig. 2) but not in primates³. In a *Saimiri* skull of the same size (Fig. 2), increase in tooth-row width measured across the alveoli from canine (2.5) to first molar (3.6) is slightly >1.4 times, and from first premolar (2.9) to first molar, nearly 1.3 times or roughly average for the more nearly parallel-sided eutherian tooth rows of platyrrhines³. The longer alveolar length in *Dolichocebus* ($c-m^2 = 17.6$) as compared with that of *Saimiri* (14.1, Fig. 2) reflects the greater dolichocephaly. Evidently, cranial form and dental system of *Dolichocebus* had already diverged widely from those of other known primates extant or extinct.

Other features not mentioned also point to the isolated position of *Dolichocebus* among known primates. They are discussed and illustrated elsewhere (P.H., in preparation). It appears, nevertheless, that Rosenberger's attribution of close relationship between *Dolichocebus* and *Saimiri* is dependent on an equivocal character which even if proven valid would not support the claim.

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ROSENBERGER REPLIES—On the advice of the referee, I deleted the following technical comment from the final manuscript¹: Cleaning of the matrix which filled the orbits of *Dolichocebus* was facilitated by periodically examining the skull under UV light. This fluoresced fossilized bone and differentiated it from exogenous material, therefore lessening the chance of damaging the specimen. After consultation with preparators and palaeontologists, I decided that it would be prudent to leave a small fenestra-like gap in the middle of the matrix sheet which closed the space between the orbits, thus protecting the true perimeter of the fenestra. This preserved its original appearance near the orbital apex. Further discussion of *Dolichocebus*, including a metrical demonstration of its uniquely narrowed interorbital process, is presented elsewhere². Note, however, that the pinching and lengthening of the process are patterned attributes correlated with closure of the interorbital sinus. Among living platyrrhines, the combination is seen only in *Saimiri*, which has further

persuaded me that a fenestrated condition was, and still is, the most likely interpretation for the fossil.

The other points raised by Hershkovitz do not require discussion. However, a contrary scientific opinion carries the burden of providing supporting evidence for an alternative solution. If the unfolding of platyrrhine evolution conformed to the darwinian axiom of 'propinquity of descent', does not *Dolichocebus* have to have close living relatives?

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Effect of cytochalasin D on thrombin-induced actin filaments in platelets

IN a recent letter¹ it was shown that cytochalasin D inhibits and reverses the formation of thrombin-induced actin filaments in platelets. It was noted also that SDS-polyacrylamide gels of reduced samples of Triton-insoluble residue from thrombin-treated platelets showed additional protein bands having molecular weights (M_r) of 51,000 and 54,000—it was suggested that these bands were tubulin. Later exchange of information between our two groups led all of us to conclude that these bands are probably reduced chains of fibrinogen or its derivatives, formed by the action of thrombin on platelet fibrinogen. An additional fibrinogen-derived band of M_r 66,000 seems to have been obscured by the bovine serum albumin added to the platelet suspension medium.

Further experimental work to confirm these conclusions is now in progress, but we wish to draw attention to this observation. We also emphasize that this finding in no way affects the main observations and conclusions of the original letter.

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BOOK REVIEWS

Who was H.J. Muller (1890–1967)?

Guido Pontecorvo

UP TO 1946 genetics contributed two Nobel Laureates: one was H. J. Muller. There are now over 20 from its flourishing modern offshoot, molecular biology. This book, the first comprehensive biography of Muller, prompted me to ask two questions. To what extent is the present generation of molecular biologists aware that Muller was the founder of their field? And do historical accounts of the development of molecular biology recognize that contribution? Two pieces of "research" gave me clear and disappointing answers.

First, I submitted the question used as title of this review to over 30 research workers of ages 30–45 in two leading research institutes perfused by molecular biology. For only very few did the name H. J. Muller ring a bell, and their answer was mainly: induction of mutations by X-rays. The overwhelming majority had never heard the name; two thought he was the inventor of the Geiger counter.

Second, I consulted three books on the development of molecular biology. James Watson, in *The Double Helix*, mentions Muller once: but not as the man who defined the genetic material, rather as one of the few prominent geneticists who, before 1953, favoured a now-untenable model of gene replication by long-distance forces of the like-attracts-like type. In his thoughtful book *The Path to the Double Helix*, R.C. Olby discusses several of Muller's contributions to that path, some prophetic and some wrong, but, in my view, misses the main point. So does Horace Judson in *The Eighth Day of Creation*. Both Olby and Judson report verbatim the famous paragraph of the 1921 Toronto paper where Muller first suggested that bacteriophages — then called "d'Herelle bodies" — could be used for a physical and chemical attack to elucidate the nature of the genetic material:

... perhaps we may be able to grind genes in a mortar and cook them in a beaker after all. Must we geneticists become chemists and physicists, simultaneously with being zoologists and botanists? Let us hope so.

This was of course an extraordinary flash of imagination which, however, Muller failed to pursue. But neither Olby or Judson reports or comments on the earlier, crucial paragraphs. In these Muller set out his most powerful theoretical idea: that there is a material, the genetic material, which has unique properties utterly different from those of any other biological

Genes, Radiation, and Society: The Life and Work of H. J. Muller. By Elof Axel Carlson. Pp.457. ISBN 0-8014-1304-4. (Cornell University Press: 1982.) \$29.95, £21.



Muller in 1927 — "the present generation of molecular biologists is unaware of how much it owes to him".

material. In current terminology these are replication, mutation, replication of mutant forms and — possibly as a later evolutionary development — coding. Muller's further generalization, again missed in those two books, was that given any material with such properties "evolution would automatically follow". At that time the suggestion of a genetic material having unique properties and being the starting point of evolution was outrageous. It prompted the chairman of the Toronto meeting, the distinguished palaeontologist H. F. Osborn, to comment: "I am glad you have a sense of humor Muller". The model proposed in the 1921 paper, Muller refined in a 1926 paper by the telling title "The gene as the basis of life". In 1965, in the last paper of his life, Muller went over the history and the development of his 1921 model which was, of course, finally vindicated by Watson and Crick in 1953.

In retrospect, the 1921 paper may well have been the pioneer step in dragging biology out of its preoccupation with metabolism and energetics — "the cell as a bag of enzymes" — towards the study of structure, function and specificity at the

macromolecular level. This movement took momentum in the late 1930s: geneticists (Muller, Timofeef-Ressovsky, Ephrussi, Waddington and Darlington) joined with physicists (Bohr, Delbrück and Zimmer) and X-ray crystallographers (Astbury and Bernal) to discuss how to attack the problem of what the genetic material is and how it works. The 1941 Cold Spring Harbor Symposium, "Genes and Chromosomes", saw this problem prominent, with Muller and Delbrück as protagonists. Schrödinger's *What is Life?*, published in 1944, stimulated further defections of physicists to genetics.

The history of later events has been abundantly recorded. Watson and Crick's definitive, exciting and powerful solution of the structure of DNA satisfied all the requirements of Muller's original model for the genetic material. To the subsequent developments — messenger, triplets, code, colinearity etc. — biochemistry, freed from "the bag of enzymes" complex, contributed decisively alongside genetics and physical chemistry. The three approaches are now one. It is for future historians to assess how much of this powerful unification had its root in Muller's paper of 1921.

The poll mentioned above suggests that the present generation of molecular biologists is utterly unaware of how much it owes to Muller. Less understandable is the failure of the three books (in the case of Watson's, my guess is that having taken Muller's postgraduate course on the gene he took it for granted that everybody else knew). Will Carlson's biography help towards a redress?

Certainly the book is quite absorbing as a review of Muller's formidable scientific contribution (over 350 papers) and as an account of a life in which an extraordinary personality went through a series of many deep hurts and a few triumphal successes, in the United States, Scotland, Germany and the Soviet Union. It reinforces, no doubt unintentionally, the picture first presented so vividly in *The Double Helix*: scientists are human beings, working in a peculiar social context.

The work that went into this biography must have been enormous: Muller kept all his manuscripts and correspondence, some 30,000 letters, now preserved in the Lilly Library of Indiana University. Most useful are the references to the life-long correspondence with his friend, the geneticist E. Altenburg. They reveal both how

Muller's mind and experiments were working and the political and academic atmosphere, including intrigues, in which they worked. Fascinating are the accounts of Muller's periods in the Soviet Union (1933–1937), where he was the most influential and active dissident against the Lysenko madness, in the Spanish Civil War, in Edinburgh (November 1937–September 1940) and, finally, in his efforts towards an international ban on atomic bomb tests. In spite of the few inaccuracies which I could spot in the Edinburgh events from first-hand knowledge, the accounts seem to be mainly reliable.

The Nobel Prize won by Muller in 1946 was awarded not for his theoretical contribution of 1921 or for his major share in clearing up the mechanism of Mendelian heredity or for turning mutation from an act of God into an event of measurable recurrence or for pioneer work on human twins, but for having demonstrated in 1926 that X-rays increase mutation rate. This,

of course, was of topical practical importance in 1946, after Hiroshima, but can be considered only as a fall-out of other work.

The increased responsibility which Muller felt after the award, made him turn increasingly to what was the preoccupation in his youth, the necessity of consciously shaping the feedback between genetics and culture for the future of mankind. His suggestion of *voluntary* germinal selection for artificial insemination by using preserved sperm produced reactions from

plain derision to violent denunciation. Time will tell.

This biography is of course essential reading for the students of the history of biology. Let us also hope that it will induce some of those young molecular biologists to read three of Muller's most significant papers¹: those of 1921, 1926 and 1965, published in 1922, 1929 and 1966. □

Guido Pontecorvo was Consultant Geneticist at the Imperial Cancer Research Fund, London, until 1980, and worked with Muller at the University of Edinburgh from 1938 to 1940.

Updating the Old Stone Age

John A.J. Gowlett

The Palaeolithic Age. By John Wymer. Pp.310. UK ISBN 0-7099-2710-X; US ISBN 0-312-59476-3. (Croom Helm/St Martin's Press: 1982.) £16.95, \$35.

HUMAN evolution as a study has a curious and persisting interest which may stem partly from the notion that had it not happened we would not be here to study it or anything else. Numerous books reflect this fascination, but most popular surveys concentrate on one side of the evidence, the biological rather than the archaeological part of the story. Although biologists (notably Julian Huxley) have explored the interplay between the biological and psychosocial aspects of human evolution, the two are still conveniently amenable to separation. Since there is little doubt that primates, ethology and early hominids have fared better in broad and popular surveys, it is time again for archaeology to do its bit. This is especially so as the standard texts of early archaeology are at least a decade out of date, now painfully lacking in references to new sites and dates.

John Wymer's *The Palaeolithic Age* thus helps to bridge a large gap, specifically, as is his intention, that between short summaries of the Old Stone Age and the specialized papers of journals. This, however, is a less easy task than it used to be: over the past 20 years fieldwork has been undertaken in many countries, and new techniques of study are developing all the time. It was just possible to be an all-round expert of the Old Stone Age, but this is no longer the case. Nonetheless John Wymer has sound credentials for his undertaking, with a wealth of excavation experience both in Europe and Africa.

Wymer's approach is to get straight on with the job, to take advantage of the natural framework of time, and to tell the story concentrating on the stone tools as the most tangible remains. Other lines of evidence are treated, but not to the same extent. He emphasizes continuity, the choice of a title for his book itself reflecting continuity with an older generation of antiquaries. The result is a useful digest of

Stone Age sites in most parts of the world, handsomely covering some areas, but undoubtedly bitty in parts, and cast in a framework more idiosyncratic than it appears at first glance.

Sound as most of the factual material is, chapter headings such as "Unspecialised Hunter-Gatherers" are highly loaded: rightly or wrongly, many archaeologists think in terms of scavenging rather than hunting for the earlier periods. "Unspecialised", too, is an assumption. If that assumption is accepted, detailed discussions about other lines of evidence can indeed be reserved for later chapters, as Wymer does.

Interesting in a modern book is the adoption in modified form of the old savagery and barbarism model, developed by Morgan and used by Marx. Childe set the transition from savagery to barbarism at the origins of food production, but Wymer sets it back many thousands of years to the Mousterian, and I can only wonder whether its continued use really aids our understanding. Then, in Wymer's framework, the Upper Palaeolithic is seen implicitly as a world-wide phase, though technically it refers to a particular technology not present everywhere. It is no fault of Wymer's that he cannot squeeze everything between these covers. Europe and Africa are done well, but the omission of the Nile sequence, and of Japan in totality, seems surprising. Highly compressed treatment of the early settlement of Australia and the Americas was probably a necessity, but could limit the appeal of the book in those areas.

These points emphasize that Wymer has provided a personal view of the Palaeolithic, rather than a standard textbook. As such it will not entirely replace previous surveys of the period, but it is without doubt a valuable complementary source for relatively detailed information about the Old Stone Age. □

J.A.J. Gowlett is at the Research Laboratory for Archaeology, University of Oxford.

¹Muller's classic papers

"Variation due to change in the individual gene" (*Amer. Nat.* **56**, 32–50; 1922). "The gene as the basis of life" (*Proc. Int. Congr. Plant Sci.* **1**, 897–921; 1929). "The gene material as the initiator and organizing basis of life" (*Amer. Nat.* **100**, 493–517; 1966). A full list of H.J. Muller's papers is in *Biographical Memoirs of the Royal Society* **14**, 349–389; 1968.

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Genes in time and in space

R.J. Berry

Ecological Genetics. By David J. Merrell. Pp.500. UK ISBN 0-582-46349-1; US ISBN 0-8166-1019-3. (Longman/University of Minnesota Press: 1982.) £15, \$25.

THIS is almost a very good book. Merrell has set out to write the sort of textbook which includes new ideas as well as regurgitated concepts and examples. In his case, the positive intention has been to consummate a union between population genetics and population ecology. As he says, this has been delayed for a number of reasons, not least because "population geneticists often assume a constant environment while population ecologists usually assume that all members of a population have identical genotypes". His explicit object is to bring together the results and concepts of ecologists and geneticists into one coherent whole.

This aim is well worthwhile: unexplained ecological variation may be largely due to genetic heterogeneity, whilst the models of geneticists need frequent tempering with muddy reality. Indeed, population biologists have caused problems for themselves because individuals, eminent and erudite though they may be, have repeatedly failed to see their organism — or population(s) — in all its relevant complexity: de Vries thought species arise through saltation (a misapprehension still being debated); the attitude of antagonists in the neutralism-selection debate of the 1970s could be predicted from their scientific background (the argument was really one between theoreticians and naturalists); life-table determination has often seemed to be an end in itself, although any life-table is dependent on environmental, biotic and genetic interactions; and so on.

Merrell has attempted to make sense of these many-sided confusions. He has not quite succeeded. One reason is that he is more familiar with *Drosophila* than the rest of the living world put together (although he has also worked with frogs), and he tends to use unadorned citations to illustrate his arguments, rather than developing relevant examples; another is his restricted choice of examples, in many cases referring to early work only. For example, *Cepaea nemoralis* is one of the few species where ecology has been fairly closely linked with genetics, but no paper more recent than 1968 is mentioned; no reference is made to speciation in *Partula*; and Hawaiian *Drosophila* is only noted in passing.

But perhaps the real reason for Merrell's failure is that ecological genetics itself may not yet have come of age; perhaps its components need to develop in their own right before they can be brought together. If so, Merrell's book may be a useful catalyst, as was Dobzhansky's *Genetics and the Origin of Species* to a previous generation. *Ecological Genetics* represents a noble

attempt at synthesis. It could be improved fairly simply by including and expanding upon more biological examples; some sections are very good (such as those on dominance and competition), whilst others ramble somewhat and need shortening (the neutralism chapter is far too long, while the evolutionary implications of MacArthur and Wilson's theory of island biogeography are not explored); and the book needs up-dating (there are references to 1978, but the main literature survey stops at 1976).

As it stands, the first edition of *Ecological Genetics* should be skimmed through by population ecologists and geneticists *sensu stricto*; but properly revised the second edition could easily become required reading for that significant part of the biological community which acknowledges the existence of a world beyond the laboratory. □

Sam Berry is Professor of Genetics at University College London. He is the author of *Inheritance and Natural History* (Collins, 1977) and *Neodarwinism* (Edward Arnold, 1982).

Chemistry at war

Alastair Hay

A Higher Form of Killing. By Robert Harris and Jeremy Paxman. Pp.288. ISBN 0-7011-2585-3. (Chatto & Windus/Hill & Wang: 1982.) £9.95, \$14.95.

CHEMICAL warfare is a subject which arouses fierce passions. Its apologists claim that it is both efficient and "humane"; it affects people but not objects, and it injures more than it kills. Victims of gas poisoning on the other hand, would probably prefer to quote evidence from an expert on the subject, Wilfred Owen:

Gas! Gas! Quick boys — An ecstasy of
fumbling,
Fitting the clumsy helmets just in time;
But someone still was yelling out and stumbling,
And floundering like a man in fire or lime . . .
Dim, through the misty panes and thick green
light,
As under a green sea, I saw him drowning.

One person who did not see gas warfare through Owen's eyes was Fritz Haber. The 1919 Nobel Prize winner in chemistry for his part in the Haber-Bosch process — the conversion of nitrogen to ammonia — Haber was also the pioneer of modern gas warfare. To him it was "a higher form of killing". This was how he described it in the acceptance speech in Stockholm after the war, and his phrase provides Robert Harris

and Jeremy Paxman with their title.

Recent allegations about the use of chemical and biological warfare (CBW) and the history of the development of such weapons form the basis of their account. The book is not a comprehensive treatise on CBW — that would be impossible to write in a single volume — it is more of an outline of the subject, except where it deals with the development of CBW in Britain during the Second World War. Here the authors have retrieved previously classified material and they deal with British war preparations in absorbing detail.

Appropriately, Harris and Paxman begin with the use of poison gas — by the German army — near Ypres in April 1915. They describe the military consequences in some detail, but any lasting impression of the use of chemical warfare in the fields of France and Flanders must be of the appalling casualties these weapons caused. Officially, 180,983 British soldiers were gassed, of whom some 6,062 died. According to the authors, however, the statistics are far from complete. They omitted numbers of men gassed in 1915; gas casualties found by the enemy; any of the quarter-of-a-million British soldiers missing in action; any soldiers who were killed in action; or even gas casualties who died after being evacuated to Britain. As for the German and Russian records, these are probably equally unreliable. In view of this, any argument about the supposedly "humane" features of chemical weapons which uses statistics from the First World War to prove that the weapons injure, rather than kill, should be treated with some caution.

The Germans may have been the first to use gas, but the Allies were not slow to respond. As the war progressed more sophisticated chemical weapons emerged from the laboratories of both sides, thousands of scientists and soldiers being recruited to screen chemicals for the ideal weapon. After chlorine, came phosgene; mustard gas followed phosgene. Because the gas was not visible, most troops failed to even don their gas masks. By the following morning most were almost blind and in the ensuing days, many died, like other cases of gas poisoning, with lungs and throat stripped of any lining and heavily congested.

During the First World War, the disadvantages of chemical weapons — problems in deploying them and fear of retaliation in kind — had become apparent. Nonetheless, in 1940, it is clear that Winston Churchill saw them as adding

A second edition of T.G.R. Bower's *Development in Infancy* has just been published by W.H. Freeman. On its first appearance in 1974, Jerome Bruner judged the work to be "one of the major books on human development to be written during the last decade" (*Nature* 252, 514; 1974). Prices of the new edition are hbk £14.80, \$20; pbk £6.90, \$9.95.

diversity to Britain's arsenal. He was keen that Britain should have adequate supplies of the weapons if only as a deterrent, to which his constant stream of memos to the Ministry of Supply to "press on" with the production of these weapons bears testimony.

All this is a matter of public record. But in their book, Harris and Paxman break new ground by claiming that Churchill was not only interested in chemical weapons as a deterrent; he was also prepared for Britain to initiate gas warfare. Had Germany invaded Britain in 1940, or 1941, the authors say that chemical warfare would probably have ensued. In addition, they claim that Churchill was convinced that chemical weapons could be used in Europe to the Allies advantage, but that this idea was firmly rebuffed by his Chiefs of Staff.

The controversial memos cited in *A Higher Form of Killing* show just how close Britain came to using CBW in the Second World War. But the fact remains that it was not. The Japanese, however, were not quite so inhibited. Recent documents published in the United States and given wide publicity in Japan are noted by the authors. These confirm that Japan conducted biological warfare against various cities in China. Further, Japanese scientists tested biological weapons in sickening experiments on prisoners of war in China in the early 1940s, the organisms involved including anthrax, cholera,

plague, hemorrhagic fever, smallpox, typhoid, typhus and gas gangrene. To study the progress of the disease some subjects were killed — with morphine — at various times and autopsied. A great deal of this research material was collected by the United States at the end of the war, for which it appears that the prosecutors did a deal with the Japanese scientists: immunity from prosecution in exchange for research data. According to a memo from Dr Edwin V. Hill, then chief of Basic Sciences at Camp Detrick in Maryland — the research centre for biological weapons in the United States — the data was invaluable because they "could not be obtained in our own laboratories because of scruples attached to human experimentation". Hill went on to say that he "hoped that individuals who voluntarily contributed this information will be spared embarrassment because of it".

In bringing their story closer to the present, the authors are inaccurate on some points. For example, on the question of the use — by the United States — of chemical agents in IndoChina, Cambodia was never heavily sprayed with defoliants; there is only one documented spraying incident. And as for the evidence that herbicide spraying led to a three-fold increase in the number of children born malformed in South Vietnam, this is still controversial. But these are relatively minor points. From the evidence cited by Harris and Paxman one needs little convincing that there are

deficiencies in the 1925 and 1972 Geneva protocols outlawing the use of chemical and biological weapons respectively.

A major flaw of both agreements is the inadequacy of their provisions for verifying that violations have occurred. The most recent allegations by the United States that the Soviet Union and her allies are waging chemical, and possibly biological warfare in Afghanistan, Laos and Kampuchea only serve to highlight the need for the protocols to be made watertight. The allegations — and they remain no more than this — are immensely difficult to verify. The Soviet Union and Vietnam deny most emphatically that they are using chemical weapons; they claim that these accusations are being used by the United States as a smokescreen to justify its own development of a new generation of binary nerve gas weapons; weapons in which the nerve agent is actually made in the shell or bomb en route to its target.

Both the Soviet Union and United States speak of the need to negotiate a treaty outlawing the use of CBW. With the Americans pushing ahead with the development of binary weapons it clearly is in everyone's interest that the bilateral negotiations between these two powers — suspended temporarily, because the United States has withdrawn its negotiators — should be successfully concluded.

Churchill had a vision of an enemy only being temporarily indisposed after a chemical attack. This was always rather optimistic and it would certainly never hold true for the current generation of weapons. Nerve gases kill; and in any chemical war in Europe most of the casualties will be civilians. *A Higher Form of Killing* will certainly help drive that message home. □

Alastair Hay is a Lecturer in the Department of Chemical Pathology at the University of Leeds.

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Plant production put in harness

J. Barber

Techniques in Bioproduktivity and Photosynthesis. Edited by J. Coombs and D.O. Hall. Pp.171. Hbk ISBN 0-08-027382-3; pbk ISBN 0-08-027383-1. (Pergamon: 1982.) Hbk \$36, £15; pbk \$16, £7.50.

THIS book, essentially a comprehensive laboratory manual, has been put together as a result of a United Nations sponsored course aimed to provide training in basic techniques for measurement of plant productivity. There are 17 contributors, all of whom were involved in teaching the course which so far has been held in India, Kenya and Yugoslavia.

The purpose of the course, and therefore of the book, is to explain various facets of the photosynthetic process with the view that these can possibly be used to predict the potential productivity of food and biomass crops. Its aim is, by practical experimentation, to pass on these concepts to students, especially those from developing countries. Because of the nature of the course, the experiments presented often use simple equipment, particularly for measurements in the field. Overall, the experiments outlined are well presented and worthwhile. Their value is enhanced by the background introductions and by useful discussions of the principles of the techniques used and methods of data interpretation.

The book falls into two parts of about equal length. Part 1 is concerned only with whole-plant photosynthesis and is subdivided into three sections. The first of these is excellent, covering a range of topics including principles of measuring solar radiation, temperature, humidity, gas exchange, stomatal conductance and plant water stress. This section also deals with the important subject of plant-growth analysis, as well as various aspects of biomass accumulation and primary production. The other two sections in Part 1 are concerned with shoot and leaf anatomy in relation to biochemical types (C_4 and C_3 etc.) and with a general survey of algal growth in terms of photosynthetic efficiency and techniques for large scale culturing.

The six sections of Part 2 contrast with those of Part 1 in that they deal with the cellular and biochemical processes of photosynthesis. As in Part 1, different experiments are presented either to demonstrate a technique or to provide evidence for a particular concept. They range from measuring photorespiration to isolation techniques for protoplasts, chloroplasts and proteins. Generally the articles are well written and introduce the reader to the principles of carbon fixation, nitrogen metabolism, electron transport and photophosphorylation.

The book should find considerable use as a teaching aid, not only at university level but even in schools. The layout of the text is attractive and the format is conducive to easy appreciation of its content. Particularly valuable are the last section, and various appendices which list extremely useful information ranging from enzyme classification to facts and figures about global and national biomass production. Although there is some inconsistency between sections with regard to the background information provided, such as sources of apparatus, and in reference to relevant publications, this book is certainly most welcome and deserves to find a large market. □

J. Barber is a Professor of Plant Physiology in the Department of Pure and Applied Biology, Imperial College, University of London.

Sweet reason in a dirty atmosphere

Tim Lincoln

The Politics of Clean Air. By Eric Ashby and Mary Anderson. Pp.178. ISBN 0-19-858330-3. (Oxford University Press: 1981.) £15, \$34.50.

THE control of pollution is only in small part a matter of science and technology. Mostly, it falls into that untidy area where practical decisions must be made from ethical judgements, and hence into the province of the politician. So it is that Eric Ashby and Mary Anderson have written a political, not a technical, history of 150 years or so of effort to tackle atmospheric pollution in Britain. Their book is all the more intriguing for that; writ large, *The Politics of Clean Air* is the politics of getting anything done.

The story they fluently relate is not simple, even when the predictable shenanigans in Whitehall and Westminster are discounted. From the first stirrings of concern early in the nineteenth century, the movement towards cleaner air has had two distinct threads. One is the control of smoke, the other control of "noxious vapours" (gases from industrial processes). The authors make plain the historical reasons for this at-first-sight puzzling dichotomy: noxious vapours could be monitored quantitatively, smoke could not; and whereas noxious vapours were emitted by a relatively few manufacturers, smoke poured from thousands of homes. The upshot was that smoke became a local responsibility (albeit subject to Parliamentary dictum) and noxious vapours that of a national body, the Alkali Inspectorate.

In 1819, London's dreadful pother was first brought to the attention of Parliament by Michael Angelo Taylor. Ensuing events took a course that was to become familiar over the next century: attack on and emendation of the Bill, and the eventual emergence of a sickly child of legislation, stunted by its passage through Parliament. Nonetheless a principle had been established: clean air was of public concern.

A second, more common, course was the introduction of a well-intentioned Bill and its subsequent death within the precincts of Westminster. On the headstone of each hindsight has chiselled the obituary, "victim of the unripe time". Here, the honours for perseverance in the face of continual disappointment must go to the "endlessly patient, calmly reasonable, consistently courteous" Lord Stratheden and Campbell. He presented his first Bill in 1884, aimed at restricting what by then was the main source of smoke pollution, the domestic fire; it foundered. So did his subsequent nine attempts.

The authors chart the advance of smoke control, two steps forward, one back, both inside and outside Parliament: the advent

and increasing political sophistication of clean air lobbies; the slow-growing awareness of industrialists that inefficient combustion in furnaces is an avoidable expense; and, above all, the weaning of the Englishman from his "pokeable, companionable fire" and "benumbed acceptance of . . . smoke as the inevitable consequence of civilization". But the process was slow and dependent on the maturation of public opinion. Only in the 1950s, marked by the murderous smog of December 1952 and the subsequent Clean Air Act of 1956, was smoke pollution brought under control.

More than anything else the history of pollution control in Britain is the history of higgledy-piggledy, but ultimately effective, development of constraints on the emission of noxious vapours; and so the history of the Alkali Inspectorate, which the book nimbly traces through a tangle of dates and characters. The Inspectorate was set up in 1864. It was born in express response to the devastation caused by "the monster nuisance of all" — muriatic (hydrochloric) acid emanating from alkali works. "In the event", say the authors, "it led to a style for the politics of pollution-control in Britain which we have followed ever since".

That style is one of sweet reason, compromise and consent, "best practicable means" (bpm), by which the Inspectorate worked (and works) as teacher and advisor to industry, not as policeman. The way of the world should dictate that control through gentle guidance, gloves on, should not work. Indeed, if prosecutions are a measure of the effectiveness of a policy, then bpm did not; for instance, during the 26-year tenure of one Chief Inspector, from 1929 to 1955, only two offenders were taken to court. The verdict of *The Politics of Clean Air* is to the contrary, however: it was precisely because the Inspectors could play friends to industry that they won the co-operation of the manufacturers, and thus most of the battles in a century of war against atmospheric pollution.

Apart from a final chapter entitled "Assessment", and brief asides on the contemporary issues of pollution from automobiles and acid rain, the book ends in 1976. In January of that year The Royal Commission on Environmental Pollution (of which Eric Ashby was the chairman) recommended that the converging tracks of pollution control policy, for smoke and for noxious gases, should become one under the authority of a national pollution inspectorate. Also, with a caveat as to its remoteness and poor record of public relations, the Commission vindicated the work of the Inspectorate and urged a continuation of best practicable means as "inherently superior to control by nationally-fixed and rigid emission standards".

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The Politics of Clean Air is avowedly a history and as such a success. It is sober and (to the credit of Mary Anderson) well-documented, yet at the same time is remarkably readable. Only when dealing with two of their themes do the authors drop the dispassionate air of the historian, and then perhaps intentionally. First, between the lines of the book runs respect — even affection — for the dogged chemists of the Inspectorate in their uncomfortable role as mediators between the interests of public, industrialists and civil servants. Second, in a restrained way the book is a campaigning document for bpm and thus pertinent at a time when the European Economic Community is applying the contrary policy of presumptive standards. The “calmly reasonable” Lord Stratheden and Campbell failed, but the policy of bpm which embodies that virtue has brought us a long way. Latter-day environmentalists might wonder, however, whether it is far enough and fast enough, and whether the policy will survive future international strains. They are also likely to doubt that one of Stratheden and Campbell’s other virtues, endless patience, is a quality appropriate for their cause in the 1980s. □

Tim Lincoln is the Book Review Editor of Nature.

An unearthly air

Conway Leovy

Weather and Climate on Planets. By K.Y. Kondratyev and G.E. Hunt. Pp.755. ISBN 0-08-026493-X. (Pergamon: 1982.) \$95, £50.

AFTER a decade of remarkable activity, a pause in planetary exploration is occurring, notwithstanding the forthcoming Galileo Jupiter orbiter-probe mission, the Large Space Telescope and the possibility that other planetary missions now under consideration may eventually fly. To some extent this pause is a consequence of the successes which have been achieved; to obtain more results of comparable scientific value while retaining wide popular interest will require extraordinary imagination and further technological innovation. Consequently, it is unclear whether the current hiatus will be followed by renewed efforts to explore the planets, or whether it really signals the declining phase of this civilization’s ambitious Solar System exploration venture.

In either case, the pause marks a period of assessment and synthesis of the results obtained so far. Such a synthesis is well underway and has already led to a new understanding of the factors which govern the formation of planets, and the present states and evolutionary histories of their

interiors, surfaces and atmospheres. A clearer picture of the uniqueness of planet Earth is emerging. In this context, *Weather and Climate on Planets* is timely.

An introductory chapter begins with a descriptive overview of the setting and properties of the planets and of theories of the origin and evolution of their atmospheres. But the major part of the book, the remaining three chapters, consists of separate self-contained reviews of the atmospheres of Venus, Mars and Jupiter. Aside from Earth, these planets have the most intensively explored and best understood atmospheres. Moreover, they are representative of the broad range of planetary atmospheres: the first two are terrestrial planets with very thick and very thin atmospheres respectively, predominantly composed of CO₂, the last is a giant planet with an atmosphere of nearly solar composition.

In the book each atmosphere is treated comprehensively, with detailed discussions of composition, distributions of temperature and density, cloud processes, circulation and, to a more limited extent, upper atmosphere aeronomical processes. Appropriately, the bulk of the text is devoted to the findings of the most important planetary spacecraft missions of the past decade: Venera 8–12, Pioneer Venus, Mariner 9, Viking 1 and 2, Pioneer 10 and 11, and Voyager 1 and 2.

Unfortunately, the book does not live up to its promise, largely because the authors do not discriminate clearly between superseded models or interpretations and later, more definitive, measurements. The result is a monograph that is long, unnecessarily repetitive and too often confusing. The treatment of argon on Mars can serve to illustrate this point. The Martian argon concentration is given in composition tables in at least four different places. In three of these, it is given variously in the range 15 to 50 per cent. A fourth table does present the definitive Viking measurement (1.6 per cent), and the careful reader could conclude that this is the correct value. However, a few pages later, confusion is re-introduced in the discussion of a pre-Viking model of the Martian volatile inventory, based on the erroneous argon concentration of 28 per cent.

Overall, the book appears to have been constructed too hastily by the publisher as well as the authors. The number of textual errors, improperly printed or inadequately annotated figures and tables, and erroneous references is more than just annoying. Although it provides a comprehensive literature survey which will be helpful for specialists, and I find the rather complete survey of the Russian language literature to be particularly welcome, the non-specialist is likely to find this book heavy going. □

Conway B. Leovy is in the Department of Atmospheric Sciences at the University of Washington, Seattle.

ANNOUNCEMENTS

Awards

Ryoichi Sasakawa (Chairman of the Japan Shipbuilding Industry Foundation) has been awarded the United Nations Peace Medal.

The award of the International Organisation of Plant Breeders for the Protection of Plant Varieties, ASSINSEL, for scientists whose basic research work has made a considerable contribution to the improvement of plant breeding methods for the benefit of agriculture and horticulture has gone to **Dr C.N. Law** (UK) and **Dr Ir. J.E. Parlevliet** (Holland).

George Weber (Laboratory for Experimental Oncology, Indiana University School of Medicine) has received the 22nd G.H.A. Clowes award of the American Association for Cancer Research. Dr Weber has also recently been given an Honorary Degree by the University of Chieti, Italy.

The Linnean Society of London has made the following awards: the Linnean Medal for botany to **Prof. Peter Davis** (Royal Botanic Garden, Edinburgh); the Linnean Medal for zoology to **Dr Humphry Greenwood** (Natural History Museum, South Kensington); the Bicentenary Medal to **Dr Lionel Higgins** the H.H. Bloomer Award; the Trail Crisp Award to **Dr John Pettitt** (Natural History Museum, South Kensington).



Prof. Dr Charles Weissmann (Zürich University, Switzerland) has received the Dr H.P. Heineken prize by the Royal Netherlands Academy of Arts and Sciences. It consists of Dfl 200,000 (£40,000) and a crystal and is (in monetary terms) the biggest scientific award in the Netherlands. It is awarded every three years for outstanding performances in biophysics, biochemistry and related sciences.

The Geological Society has made the following awards: The Wollaston Medal to **Prof. P.J. Wyllie**; The Lyell Medal to **Prof. G.P.L. Walker**; The Murchison Medal to **Prof. D. Flinn**; The Wollaston Fund to **Dr H.C. Jenkyns**; The Murchison Fund to **Dr R.B. Richards**; The Lyell Fund; one moiety to **Dr D.G. Roberts**, one moiety to be shared by **Drs P.W. Francis and R.S. Thorpe**; President's Awards to **Dr R.J. Knipe** and **Dr C.H. Donaldson**; The R.H. Worth Prize to **Dr A.E. Mourant**.

The Lister Institute of Preventive Medicine have announced the following research fellowships: **Dr Judith P. Armitage** (Microbiology, University College London); **Dr G. Marius Clore** (National Institute for Medical Research, Mill Hill); **Dr Alec J. Jeffreys** (Genetics University of Leicester); **Dr Sai-Kit A. Law** (Biochemistry, University of Oxford); **Dr Stephen J. Perkins** (Kennedy Institute of Rheumatology, London).

Dr Richard Axel (Institute of Cancer Research at Columbia University) has received the NSF's seventh annual Alan T. Waterman Award for his novel method of introducing virtually any gene into mammalian cells.

The Ernst Jung Prize for Medicine has been awarded to **Prof. Hartmut Wekerle** and **Prof. Rolf M. Zinkernagel** (Institut für Pathologie der Universität Zürich).

Biotrol, the French manufacturers of specialized control sera and colorimetric assays, are offering a prize (10,000 FF) for the best research work which could result in the introduction of new laboratory diagnostic tests. Contact: Bill Daring, Burgess, Daring Associates, Manchester, UK.

Churchill Travelling Fellowships are open to all UK citizens of any age or occupation, and since no educational or professional qualifications are needed, they are of special interest to people who would not be eligible for other types of grants. The object of the awards is to enable those who would not otherwise have a chance, to gain a better understanding of the lives and work of people in countries overseas, and to bring back useful knowledge, skill and experience for the benefit of our community. Grants are offered in different categories each year. The only requirement is that applicants have to show that they can make effective use of the opportunity both while they are abroad and when they return. To apply send your name and address only on a postcard between August and mid-October to the Winston Churchill Memorial Trust, 15 Queen's Gate Terrace, London SW7, UK.

The Clarke Institute of Psychiatry Annual Research Fund Award is awarded annually to a clinical or basic scientist who has published a report or dissertation on outstanding research within the field of mental health conducted during the preceding year. The scientist must have carried out his work in Canada while resident there. Applications and nominations to G.D. Carpenter, Research Fund Committee, Clarke Institute of Psychiatry, 250 College St, Toronto, Ontario, Canada M5T 1R8.

Universities, academies, educational institutions and research centres are invited to nominate candidates for the King Faisal International Prize to be awarded in the field of physics in *Rabi al-Awal 1403 A.H.* (January 1983). A nominee must have accomplished outstanding academic work in physics, that must have been published, for the benefit of mankind. An attached Arabic abstract is preferred if the work is published in any other language and must be original. The nominations must give full particulars of the nominee's academic background, experience and/or publications, copies of educational certificates and three 6×9 cm photographs. Nominations and 10 copies of the work are to be sent by registered airmail to the General Secretariat, King Faisal International Prize, PO Box 352, Riyadh, Saudi Arabia before 7 August 1982.

Through the Irish Foundation for Human Development, the annual Curt P. Richter Prize in Psychoneuroendocrinology has been established in recognition of meritorious research. The prize will be awarded annually for the best essay or manuscript (original research or a review including original research) submitted by a scientist or physician under 40 years of age by 1 January 1983. Manuscripts to: Dr G. Langer, Allgemeines Krankenhaus der Stadt Wien, Psychiatrische Universitätsklinik, Lazarettgasse 14 A-1097 Vienna, Austria by 2 January 1983.

Sakharov scholarship

The Sakharov International Committee Inc., a Washington-based human rights organization whose board includes 29 Nobel prize winners has launched a fund to award scholarships to qualified students "dedicated to the pursuit of Sakharov's scientific or humanistic goals" and prizes to scientists who "in addition to outstanding achievements in the fields are also deeply devoted to the cause of human liberty". The fund will also assist scientists persecuted for their advocacy of human rights. The fund is being raised by a direct appeal to individual scientists and scientific organizations. The committee's address is PO Box 9422, Washington DC 20016, USA.

CORRESPONDENCE

Continued from page 114

cells to be tested in humans without the whole range of present toxicity study requirements. This may perhaps be part of Weatherall's "unattractive message that the experts must tell the public" and does fall into the category of "desperate remedies for desperate ills": it has also served to reactivate industrial interest in this therapeutic area.

An even more delicate area was not discussed. Proposals⁹ for attaining a population of whom 95 per cent may use synthetic mood-modifying drugs regularly by the 1990s may be becoming more tangible with the latest developments in our biochemical understanding of the already overprescribed 1,4-benzodiazepines: identification¹⁰ of a specific receptor for them, modifications of the endogenous ligand(s)¹¹ of the benzodiazepines and the subclassification of those receptors¹³ with their own agonist/antagonist complement such as the type I-selective triazolopyridazines,¹⁴ exposes a whole new area for exploitation into social psychotropic drugs. The experts ought to be talking about some of these aspects also, accepting Sakharov's charge¹⁵ that we the scientists should take responsibility for the applications of science and technology to life.

C. UPTON

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Return of malaria

SIR — Chapin and Wasserstrom¹ suggest that resurgence of malaria in Central America and India was the result of introduction of high yielding varieties (HYV) of rice and cotton. We feel that this conclusion is incorrect and that the basic data used by Chapin and Wasserstrom were defective. What follows is

our view of how malarial resurgence came about in India.

(1) During the years of resurgence cotton production remained at a roughly constant level. There was a marginal increase in rice production but the area under HYV was low. Use of DDT in 1960–64 was 73,313 tonnes in public health as against 3,000 tonnes in the agricultural sector (see table). Even in subsequent years, use of DDT in agriculture was far less than for public health. However, it may also be pointed out that malathion insecticide has been in continuous use in agriculture since 1958 and malaria vectors have remained susceptible to it wherever it has not been used by the National Malaria Eradication Programme (NMEP). It was used in Gujarat to control DDT and HCH resistant *Anopheles culicifacies* and between 1970 and 1973, susceptible *A. culicifacies* developed 4-fold resistance².

(2) The origins of the setback to the Indian anti-malaria programme can be traced to 1963 when approximately 2 million of the population were involved in scattered focal outbreaks in the consolidation phase. Focal outbreaks during subsequent years became more serious. In areas which could not be tackled an estimated population of 12, 17 and 32 million respectively were temporarily reverted to attack phase during 1965, 1966 and 1967. During 1968, out of a total of 393.25 units, 71.385 units involving a population of 91 million were reverted to spraying, 51.785 from the consolidation and 19.60 from the maintenance phase. In addition, there were 41.60 units under NMEP which never moved out of the attack phase. These units were located in hyperendemic areas of 20 states, union territories and the coalfields^{3,4}.

(3) Whilst the incidence of malaria in rural areas went down, there was a resurgence in the cities of India. In Tamil Nadu in 1963, 95 per cent of the cases came from urban areas. In 1964–67 urban malaria constituted 80 per cent of the problem, and malaria was found to be diffusing towards the rural areas⁵. *A. stephensi*, the urban malaria vector, was found to be resistant to DDT and HCH.

(4) There are 7 major vectors of malaria in India. Insecticide resistance has been detected in *Anopheles culicifacies* and *A. stephensi* with isolated reports in *A. fluviatilis*^{6,7}. The first report of increased tolerance in *A. stephensi* came from Erode, Tamil Nadu in 1956⁸ and that in *A. culicifacies* from Gujarat in 1957⁹. Between 1959 and 1967 DDT resistance in *A. stephensi* and *A. culicifacies* became widespread. During 1967–69 there were 96 units under persistent attack phase and malaria incidence increased in 56 units. In 44

units tested, *A. culicifacies* was found resistant to DDT in 35 units and to DDT + HCH in 5 units. *A. stephensi* was also tested against DDT in 8 units and was found to be resistant in four (ref. 10). In fact withdrawal of spraying has been reported to have resulted in reversion towards susceptibility. Thus resistance was the result of spraying under NMEP and it preceded the introduction of HYV in India.

We believe that resurgence of malaria in India was largely due to administrative, social, economic and financial reasons¹¹, and its diffusion was facilitated by the widespread occurrence of the parasite and anophelism in the country. The resistant populations of these mosquitoes found additional breeding grounds due to development activities. In order to stabilize and increase agricultural production, the gross area under irrigation was increased from 29.05 million hectares in 1960–61 to 37.10 in 1968–69, steadily rising to 57.02 million hectares in 1979–80¹². Similarly, unplanned growth in urban and rural housing and lack of adequate water disposal facilities made the surroundings more mosquito-genic and receptive to malaria. Large areas of the country (such as the Punjab) which in the past were prone to malaria epidemics in years of high precipitation turned endemic. Irrigation increased the average humidity of the atmosphere and made the regions more conducive for mosquitoes' survival. This had the most profound effect on the basic reproduction rate. Thus there was a resurgence of malaria in areas of the country at one time freed from the disease.

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Incidence of malaria, DDT used and agricultural production in India

	1960	1965	1966	1967	1968	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978
Malaria incidence (millions)	—	0.10	0.15	0.28	0.27	0.35	0.69	1.31	1.43	1.93	3.17	5.16	6.46	4.74	4.14
DDT (tech) used in public health (tonnes)	21,007	6,671	2,762	3,045	5,821	6,401	6,205	7,350	7,034	6,821	6,700	7,250	7,250	9,051	6,800
DDT (tech) used in agriculture (tonnes)	600	2,400	2,400	2,400	2,400	2,400	2,400	2,400	2,400	2,880	2,934	2,450	3,000	2,450	4,720
Rice production (million tonnes)	34.50	30.59	30.44	37.61	39.61	40.43	42.23	43.07	39.25	44.05	39.58	48.74	44.91	52.67	53.77
Area under rice in million hectares (in parentheses, under HYV)	34.1 (0)	35.5 (0)	35.3 (0.9)	35.4 (1.8)	37.0 (2.7)	37.6 (4.3)	37.6 (5.6)	37.8 (7.4)	36.7 (8.2)	38.3 (10.0)	37.9 (11.2)	39.5 (12.4)	38.5 (13.3)	40.3 (16.1)	40.5 (16.9)
Cotton production (million bales)	5.60	4.85	5.27	5.78	5.45	5.56	4.76	6.95	5.74	6.31	7.16	5.95	5.84	7.24	7.96

Source: NMEP, IARI & Fertilizer Statistics, Fertilizer Association of India (1980–81). Accurate figures for DDT used in agriculture (1960–72) are not available.